A STUDY OF THE RELATIONSHIP BETWEEN

VIRUS AND HOST CELL

by

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A thesis submitted for the degree of Doctor of Science

at the University of Edinburgh

March - 1952

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INTRODUCTION

Since viruses were first recognized as infectious agents the mechanism of their multiplication has been one of the most intriguing and puzzling problems of biology. A variety of explanations has been advanced to account for the increase in virus material when a susceptible cell is invaded by a virus particle. For many years it was assumed that they were micro-organisms which penetrated the host cell and multiplied there by a method of fission in a manner similar to that of bacteria. The viruses were thus thought to be endowed with the properties of living entities. Doubt was thrown on this concept by the finding of Stanley in 1935 that tobacco mosaic virus could be obtained in a crystalline form and that it had the chemical and physical properties usually associated with large protein molecules. During the ensuing years a good deal of discussion and speculation on the nature of viruses took place. Various theories were proposed, in one of which it was postulated (Stanley, J. Phys. Chem. 42, 55, 1938) that viral multiplication was a self-reduplication or autocatalytic process. Northrop (J. Gen. Physiol. 23, 59, 1939) suggested that a virus precursor was present in the cell before infection and this was transformed into virus by an autocatalytic process. These suggestions led to no clarification of the problem since

the mechanisms invoked are scarcely less mysterious than viral multiplication.

In the meantime Stanley's purification of tobacco mosaic virus led to similar work on other viruses. In a decade of intense activity a number of plant, animal and bacterial viruses was obtained in a highly purified state and subjected to detailed chemical and physical analysis. (Beard, J. Immunol. 58, 49, 1948). All the purified viruses so far investigated have been found to contain protein and nucleic acid. With some viruses such as the E. coli bacteriophages these appear to be the only constituents of the infectious particle; others such as influenza and equine encephalomyelitis contain phospholipid and neutral fat in addition. Although influenza virus appears to have a mucinase closely associated with the elementary body (Burnet, Aust. J. Sci. 10, 21, 1947) none of the enzyme activities normally involved in respiration and energy transfer have yet been found in a virus (Stanley and Knight, Les Virus, études biochimiques et biophysique récentes, Fondation Franqui, 1945, Bruxelles; Bauer, Nature, 164, 767, 1949). Thus at the end of World War II there was a considerable amount of knowledge available about the growth, purification and chemical and physical properties of several viruses, and a vast literature on the biological properties of many different viruses (van Rooyen and Rhodes, Virus Diseases of Man, Nelson 1948; Bawden, Plant Viruses and Virus Diseases, 1943; Delbrück,

Biol. Rev. Cambridge Phil. Soc. 21, 30, 1946; Pirie, Ann. Rev. Biochem. 15, 573, 1946) but little was known about the course of events which lead to the formation of new virus particles within the infected cell.

The author began work in the virus field at the University of Edinburgh in 1946 in collaboration with Dr. C. E. van Rooyen. Interest was first centred in the problem of obtaining fowl pox virus in a highly purified state and subjecting it to chemical and physical analysis. After some months of work it became apparent that the most interesting and fundamental problem in virology was the process of viral multiplication, that this was a study in biochemical dynamics and that the collection of analytical data for another purified virus was not likely to contribute much to its solution. At that time the view that virus multiplication was similar to that of bacteria was still widely held, but it seemed to the author that this hypothesis was open to doubt.

While most bacteria have the complete metabolic apparatus which will enable them to grow in a liquid medium often remarkably simple in composition, and can adapt themselves to grow under a variety of conditions, no virus has yet been found to grow in the absence of its host cell. Since virus particles apparently have none of the metabolic functions required for growth, it followed that during their intracellular growth the host must play more than a purely passive rôle. The idea was then conceived that the virus

attached itself to the host and from that time on directed the course of intracellular reactions. Under this influence the cell was supposed to cease its synthesis of cell constituents and instead synthesize new virus. The onus for virus production was thereby placed entirely on the cell and the invading particle took little active part after initiating the infection. While this was a considerable departure from prevailing views, there were already indications in the literature that such a mechanism had been considered by others (Green, Science, 82, 443, 1935; Laidlaw, Virus Diseases and Viruses, Cambridge Univ. Press, 1938). It is now known that a number of research groups in Europe and North America were working with a similar hypothesis in mind at the end of 1946 and, as will be shown later, this hypothesis in a more elaborate form has now gained wide acceptance.

Since there was little information available on the metabolism of virus infected cells, the scheme was purely hypothetical. It was, however, theoretically reasonable and what was most important suggested a number of new experimental approaches to the problem of viral multiplication. Thus, one possible line of attack would attempt to incorporate radioactive isotopes into the virus particle, and fortunately the wide availability of isotopes immediately after World War II made this approach a feasible one. If the growing virus could be labelled, it should then be possible to trace the pathways of labelled compounds through their various metabolic reactions into the virus and thereby gain some know-

ledge of viral synthesis. Utilizing a labelled virus, it would also be possible to learn something of the fate of the particle upon infection of the cell. This was a problem which particularly intrigued the author since there were suggestions in the literature that the virus particle could not be recovered by breaking open the cell shortly after infection. Stanley (J. Gen. Physiol. 25, 881, 1942) had, in fact, succeeded in labelling tobacco mosaic virus with P^{32} and found that after infection of plants with the labelled virus a large amount of the isotope was no longer associated with virus. However, he did not develop this approach.

Work was begun by the author in 1947 at the University of Toronto to determine whether it might be possible to label an animal or bacterial virus with P^{32}. If this proved successful, it was proposed firstly to study the behaviour of the labelled virus particle on infection of its host and secondly to investigate intracellular virus synthesis utilizing isotope techniques.

The main technical requirements in such a study were (a) a virus-host system which was relatively simple and subject to experimental control; (b) a virus that could be grown readily in high yield, was easily purified from host constituents and whose chemical and physical properties had already been worked out in some detail; and (c) a virus that contained one per cent or more of phosphorus so that it would take up sufficient P^{32} during growth to make the labelled

virus a useful experimental tool. Two systems satisfied these requirements, (1) influenza virus adapted to growth in the embryonated egg and (2) the seven T bacteriophages active on Escherichia coli. Work was therefore initiated on both systems.

It was found that influenza virus could be labelled with P^{32} by growing it in the allantoic membrane of the embryonated egg with inorganic P^{32} placed in the allantoic fluid. Control experiments and chemical analysis indicated that the label was incorporated into the virus during its growth, and there was no direct exchange of P^{32} between virus and inorganic phosphate. Chemical analyses of the phosphorus constituents in allantoic membrane were also carried out in preparation for experiments on membrane infected with labelled virus.

The remainder of this thesis is concerned with research on the set of seven bacteriophages, active on the host E. coli, commonly referred to as the T group. When the present work was begun more exact knowledge had been accumulated about the biological properties of bacteriophages than any other type of virus. Bacteriophages are relatively easy to handle and had attracted the attention of a number of outstanding workers among whom d'Herelle, Burnet, Craigie and Delbrück were pre-eminent.

Thus for the seven T coliphages which had been studied intensively (Delbrück, Biol. Rev. Cambridge Phil. Soc. 21, 30, 1946; Anderson, Bot. Rev. 15, 464, 1949) it was known that T2, T4 and T6 were sperm-like in shape, with

heads about 100 mμ in diameter and tails 115 mμ in length, and serologically related. T3 and T7 were spherical, about 45 mμ in diameter and related serologically while T1 and T5 had the same shape as the even numbered phages but were somewhat smaller and not related serologically to each other or any other member of the group. T2 phage was readily purified in quantity and was known to consist of 60% protein, 40% desoxyribonucleic acid and a trace of fat. All the T phages could be rapidly assayed with an accuracy of better than 15%. The phages, in fact, appear to be unique among viruses in the ease with which they may be assayed. The quantitative estimation of animal viruses on the other hand is a procedure usually lengthy, inaccurate and often extremely costly.

When added to a broth culture of susceptible cells the T phages adsorb to the cells at a rate dependant upon the relative concentration of cells and virus particles and upon the salt concentration of the medium. Further, the adsorbed particles are distributed among the cells in accordance with the Poisson law and it is an easy matter to calculate the proportion of cells infected with a given number of virus particles. Up to several hundred virus particles may adsorb to a single cell. When sufficient virus has been added to ensure that each cell has adsorbed more than one particle a culture is said to be multiply infected. Single infection refers to the case in which each infected cell has adsorbed only one virus; since less than one virus particle per cell must be added, a proportion of cells in a singly infected culture

is not infected. When the cell is infected a "latent period" follows during which intracellular multiplication of virus occurs. The length of this period is characteristic for each phage and varies from 13 minutes with T1, T3 and T7 to 40 minutes with T5. At the end of the latent period the cell lyses and releases the new phage into the medium. The average number of new particles formed per cell, the "burst size", is also characteristic for each phage and varies from 150 for T2 to 300 for T3 under the conditions of "one step growth" experiments (Delbrück, Advances in Enzymology, 2, 1, 1942). The burst size is, however, greatly influenced by the conditions of infection, physiological state of the host, and the composition of the medium. Even under the best physiological conditions the burst sizes of individual bacteria may vary from less than 10 to more than 1000 particles (Delbrück, J. Bact. 50, 131, 1945) after infection with T2 phage.

Each of the T phages can mutate during growth in a number of known ways and probably a variety of unknown ways. The host bacteria also mutate and it is relatively easy to obtain variants of the original strain resistant to one or more of the seven phages. Some of the mutants of both phage and cells are extraordinarily useful as experimental tools in biochemical experiments and the problems involved in the mutation of the phages provide the main approach to the genetic side of the problem.

As described in the present work, it was found that T2 bacteriophage could readily be labelled with P^{32}

and that at least 95% of the isotope was contained in the desoxyribonucleic acid moiety of the virus. When the cell was infected with the labelled virus there was a rapid and extensive breakdown of the particle although up to 35% of the P^{32} appeared in the viral progeny. The extent of breakdown and the contribution of parental P^{32} to progeny varied considerably with experimental conditions and were studied in some detail. In a later section the significance of these findings is discussed in the light of recent knowledge on virus multiplication.

EXPERIMENTAL

This section includes the following papers:

1. Uptake of Radioactive Phosphorus by Influenza Virus, by A. F. Graham and Laurella McClelland, Nature 163, 949, 1949.

2. The Uptake of Radioactive Phosphorus by Influenza Virus A (PR8 Strain), by A. F. Graham and Laurella McClelland, Can. J. Research E, 28, 121, 1950.

3. The Chemical Analysis of Purified Influenza Virus A (PR8 Strain) containing Radioactive Phosphorus, by A. F. Graham, Can. J. Research, E, 28, 186, 1950.

4. The Fractionation of Phosphorus containing Constituents in the Allantoic Membrane of the Embryonated Egg, by A. F. Graham, Can. J. Research E, 28, 271, 1950.

5. The Toxicity of P^{32} for Normal and Influenza Virus Infected Embryos, by A. F. Graham, G. Dempster and Barbara Buchner, J. Bact., in press, March 1952.

6. Breakdown of Infecting Coliphage by the Host Cell, by S. M. Lesley, R. C. French and A. F. Graham, Arch. Biochem. 28, 149, 1950.

7. Studies on the Relationship between Virus and Host Cell I. The Preparation of T2r+ Bacteriophage Labelled with Radioactive Phosphorus, by S. M. Lesley, R. C. French and A. F. Graham, Can. J. Research, E. 28, 281, 1950.

8. Studies on the Relationship between Virus and Host Cell II. The Breakdown of T2r+ Bacteriophage upon Infection of its Host Escherichia Coli, by S. M. Lesley, R. C. French, A. F. Graham and C. E. van Rooyen, Can. J. Med. Sci. 29, 128, 1951.

9. Studies on the Relationship between Virus and Host Cell III. The Breakdown of P^{32} Labelled T2r+ Bacteriophage Adsorbed to E. Coli Previously Infected by Other Coliphages of the T Group, by R. C. French, S. M. Lesley, A. F. Graham and C. E. van Rooyen, Can. J. Med. Sci. 29, 144, 1951.

10. Studies on the Relationship between Virus and Host Cell IV. The Contribution of Phosphorus from P^{32} Labelled T2r+ Bacteriophage to its Progeny, by R. C. French, S. M. Lesley, A. F. Graham and C. E. van Rooyen, J. Bact. in press.

11. Studies on the Relationship between Virus and Host Cell V. The Breakdown of T2r+ Bacteriophage by A. F. Graham, R. C. French, S. M. Lesley, and C. E. van Rooyen, to be published, Can. J. Med. Sci.

12. Studies on the Relationship between Virus and Host Cell VI. The Fate of T2 Bacteriophage Inactivated with Ultra-Violet Radiation, by A. F. Graham, R. C. French, S. M. Lesley and C. E. van Rooyen, to be published, Can. J. Med. Sci.

13. An Apparatus for Pipetting Radioactive Solutions by A. F. Graham, J. Lab. Clin. Med. 36, 146, 1950.

(Reprinted from Nature, Vol. 163, p. 949, June 18, 1949)

Uptake of Radioactive Phosphorus by Influenza Virus

To gain information about the mechanism of virus synthesis in the host cell, we have been carrying out experiments on influenza virus growing in the embryonated egg. It was of particular interest to determine whether virus growing in the presence of radioactive phosphorus would incorporate the isotope into its structure. Some of the results are described here.

As a preliminary to the study with phosphorus-32, it was necessary to determine the distribution of phosphorus in the virus. Purified virus for this purpose was obtained as follows. Eleven-day embryonated eggs were inoculated with 10–100 ID_{50} of influenza virus A (*PR* 8 strain) by the allantoic route. After 48 hours incubation at 36° C. the allantoic fluid was harvested and the virus concentrated and purified by adsorption on, and elution from, red blood cells, followed by two cycles of differential centrifugalization. Several preparations were examined with the electron microscope; they contained little electron-absorbing material apart from the elementary bodies and a few of the rod-like forms typical of this virus. In several such preparations, one ID_{50} for embryonated eggs contained 5×10^{-14} to 4×10^{-15} gm. nitrogen.

Extraction of dried purified preparations with a mixture of alcohol and ether (3/1) removed about 25 per cent of the total weight and 50 per cent of the total phosphorus of the virus. Most of this alcohol–ether soluble material was also soluble in petroleum ether and, in accordance with previous observations[1], was assumed to contain all the virus phospholipid. The phosphorus remaining in the alcohol–ether insoluble fraction constituted about 0·5 per cent of the virus weight: the total phosphorus content of the virus was about 1 per cent.

Previous workers[2,3] have observed that at least part of the phosphorus of the alcohol–ether insoluble residue is distributed between pentosenucleic acid and desoxypentosenucleic acid. In an attempt to separate these two acids quantitatively, the procedure of Schmidt and Thannhauser[4] was applied to the residue; about 94 per cent of the phosphorus separated with the pentosenucleic acid fraction.

In experiments with radioactive phosphorus, phosphorus-32 as sodium phosphate was inoculated into the allantoic cavity of embryonated eggs at an arbitrary interval three hours after injection of virus. In one of our experiments the radioactive solution inoculated into each egg registered 57,000 counts per minute on a Geiger–Müller counter. The allantoic fluid was harvested after about forty-eight hours, and the virus, purified by the procedure described above, had a specific activity of 6·6 counts/min./μgm. phosphorus. The phospholipid fraction registered 3·0 counts/min./μgm. phosphorus, while the alcohol–ether insoluble residue gave 10 counts/min./μgm. phosphorus. An attempt has been made to ascertain the specific activities of the pentose- and desoxypentosenucleic acid fractions separated from the alcohol-ether insoluble residue by the method of Schmidt and Thannhauser. By far the greater proportion of the phosphorus-32 was associated with the pentosenucleic acid fraction. Sufficient material has not yet been available at any one time to make a satisfactory estimate of the specific activity of the desoxypentosenucleic acid fraction.

Strong supporting evidence that the isotope was, in fact, incorporated into the virus structure was afforded by control experiments which demonstrated that there was little or no direct physical exchange *in vitro* of phosphorus-32 between radioactive sodium phosphate and the virus; nor did the radioactive virus exchange its phosphorus-32 when dialysed against phosphate buffer.

This work has been aided by a grant from the National Cancer Institute of Canada. We wish to thank Dr. C. E. van Rooyen for his interest throughout. The work is being continued, and a full report will appear later elsewhere.

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University of Toronto.
Feb. 4.

[1] Taylor, A. R., *J. Biol. Chem.*, **153**, 675 (1944).
[2] Knight, C. A., *J. Exp. Med.*, **85**, 99 (1947).
[3] Beard, J. W., *J. Immunol.*, **58**, 49 (1948).
[4] Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).

Reprinted from Canadian Journal of Research, E, 28 : 121-134. June, 1950

THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY INFLUENZA VIRUS A (PR8 STRAIN)[1]

By A. F. Graham and Laurella McClelland

Abstract

Radioactive inorganic phosphorus placed in the allantoic sacs of embryonated eggs three hours after inoculation with influenza virus was incorporated into the structure of the virus during its growth. There was little or no direct exchange between the virus and radioactive inorganic phosphorus. The specific activity of purified labelled virus rose linearly with increasing amounts of radioactive phosphorus administered to the eggs. When radioactive phosphorus was placed in the allantoic sac 48 hr. before inoculation with influenza virus the newly formed labelled virus had a specific activity about 20% higher than when isotope was administered at the same time as virus. As the amount of isotope injected into each infected egg was increased up to 775 μrd. an increasing number of embryos died during the subsequent period of virus growth. The yield of virus from the surviving eggs was not less than from eggs which had not received radioactive phosphorus. Under the experimental conditions described the amount of isotope which could be introduced into influenza virus was not sufficient to permit the use of the marked virus in metabolism experiments in animals or embryonated eggs.

Introduction

The purpose of this work was to determine whether the elementary bodies of influenza virus could be labelled with radioactive phosphorus during their growth in the allantoic membrane of the embryonated egg. If purified influenza virus could be obtained with a sufficiently high content of isotope it was considered that a number of problems in the field of animal viruses would be open to new methods of study.

Thus, in the first place, it would be of interest to determine the fate of the isotope when mouse lung or allantoic membrane was infected with radioactive virus since this might throw some light on the mechanism of cell infection by the virus. Secondly, chemical analysis of radioactive virus would show whether the isotope was concentrated in one or more of the virus constituents, indicating that these constituents played a special role in virus growth. Thirdly, it would be of great interest to compare the rate of uptake of isotope by some of the phosphorus containing constituents of the normal and infected cell; this would give an indication as to whether virus infection alters the phosphorus metabolism of the cell. Fourthly, radioactive influenza virus might be useful in immunological studies along lines suggested by the work of Libby and Madison with labelled tobacco mosaic virus (9).

Some of these problems are general to the study of all viruses, and it appeared that a suitable model system for preliminary study would be that of influenza virus growing in the allantoic membrane. Conditions for growth, methods of purification, and chemical analysis of this virus have already been worked out in detail by previous investigators. Although no published work was available on the application of isotope techniques to the study of anima!

[1] *Manuscript received December 30, 1949.*
Contribution from Connaught Medical Research Laboratories, University of Toronto, Ont.

viruses, Cohen (1, 2) and Putnam and Kozloff (11) have investigated the growth of T_2, T_4, and T_6 bacteriophages on *Escherichia coli* in the presence of radioactive phosphorus. Since these bacterial viruses readily incorporated the isotope into their structures there was a strong suggestion that influenza virus would also become labelled during its growth in the fertile egg.

The present paper describes the conditions under which radioactive phosphorus was incorporated into the structure of influenza virus. A preliminary report of this work has already appeared (5).

Methods

Determination of Total Phosphorus.

Total phosphorus was determined by the method of Kuttner and Lichenstein (8) with several modifications introduced to suit the present purpose.

An aliquot of the solution to be estimated was pipetted into a test tube graduated at the 15 ml. mark, and 1.0 ml. of 15 *N* sulphuric acid was added. The mixture was heated on an open flame until most of the water had been removed and digestion was continued on a sand bath over an electric hot plate for one hour. One drop of concentrated nitric acid was then added and the heating continued a further half hour. One to two ml. of distilled water was added and then evaporated off rapidly over an open flame. This procedure removed decomposition products of nitric acid which interfered seriously with the subsequent color development.

After addition of 2.0 ml. of 5.51% ammonium molybdate (w/v) the volume was made up to about 13 ml. with water and thoroughly mixed. To this was added 1.5 ml. stannous chloride (working solution prepared daily by diluting 1.0 ml. of stock solution, 10 gm. stannous chloride in 25 ml. concentrated hydrochloric acid, to 200 ml. with water) the tube being shaken continuously during addition. The volume was made up to 15 ml., mixed, allowed to stand 10 min. at room temperature, and the per cent transmittance read against distilled water at 630 mμ in a Coleman Spectrophotometer. The calibration curve was linear between 0 to 20 μgm. P.

Determination of Radioactive Phosphorus (P^{32})

Practically all the determinations of radioactivity were made on solutions of material in water, biological fluids, or organic solvents. The method used will be fully described (4).

Throughout this paper the activities of P^{32} sources are expressed as counts per minute (c.p.m.), that is, the number of impulses registered per minute on a Geiger-Müller counter. Radioactivity measurements were carried out in triplicate for each solution. Each source was measured with a standard deviation of 2 to 5% of the net counting rate (7) unless the net counting rate was less than about 20% of the background, when a standard deviation of 25% was considered to be sufficient accuracy. Since the method was calibrated (4) against a standard RaD + E source from the National Bureau of

Standards, the results may be converted to microrutherfords (μrd.) using the factor 1000 c.p.m. are equivalent to a β-ray activity of 85.8 μrd.

The radioactive phosphorus was obtained at monthly intervals from the National Research Council, Chalk River, as phosphoric acid in 0.005 *M* hydrochloric acid, carrier free. The decay rate of the material was checked experimentally from time to time, but, in general, corrections for decay of P^{32} during an experiment were calculated from a decay curve assuming a half-life of 14.5 days.

Determination of Protein Nitrogen

Protein nitrogen was estimated after digestion with sulphuric acid and Perhydrol by the Nessler method using the reagent described by Vanselow (16). The density of the developed brown color was determined at 460 mμ against the reagent blank. Ammonium sulphate was used as a standard, the calibration curve being linear from 0-60 μgm. N.

Determination of Infectivity of Influenza Virus

Serial tenfold dilutions of virus suspension in broth were injected in 0.2 ml. amounts into 11 day embryonated eggs by the allantoic route. Generally, at least six groups of seven eggs each were used for an infectivity titration. After incubation at 36° C. for 48 hr. a small quantity of allantoic fluid was aspirated from each egg and tested for haemagglutination with a washed suspension of chicken erythrocytes. The 50% infectivity end-point (ID_{50}) was calculated according to the method of Reed and Muench (12).

In many cases the virus content of solutions was estimated by the chicken red cell agglutination (CCA) test of Miller and Stanley (10). This test was used only as a rough indication of the amount of virus in a suspension, its reproducibility and accuracy being much inferior to the infectivity titration.

Experimental

Growth, Purification, and Properties of Influenza Virus

During the course of the work several methods for harvesting and purifying influenza virus were tried but the best preparations were obtained by the following procedure which is similar in many respects to that worked out by Taylor *et al.* (15).

Eleven-day embryonated eggs were inoculated by the allantoic route with 10-100 ID_{50} of influenza virus A (PR8 strain) in 0.2 ml. amount. After incubating the eggs for 48 hr. at 36° C. they were opened under sterile conditions and a large blood vessel of the inner chorioallantoic membrane was severed. The mixture of blood and allantoic fluid was aspirated into 250 ml. centrifuge bottles and allowed to stand at 5° C. for 18 hr. to permit complete agglutination of the red blood cells by the virus. Following centrifugation the agglutinated cells were suspended in ice-cold 0.85% (w/v) sodium chloride adjusted to pH 7.0, hereafter referred to as "saline", and again centrifuged. A volume of saline equivalent to 1/10 the original volume of allantoic fluid was then added and the virus eluted from the cells by incubating at 37° C.

for three hours. The cells were removed by sedimentation in a horizontal centrifuge at 1000 r.p.m. and the supernatant subjected to further centrifugation for 10 min. in an angle centrifuge at 2000 g. The supernatant solution thus obtained was centrifuged at 20,000 g for one hour at 5° C. in a Sorvall angle centrifuge to sediment the virus. A few drops of saline were added to the pellet after the supernatant solution was poured off. Whenever possible the mixture was allowed to stand overnight at 5° C. since the virus resuspended more readily under these conditions. The virus was then resuspended by pipetting for several minutes through a fine-tipped pipette, the volume made up to 1/100 the volume of original allantoic fluid with saline and centrifuged at 5° C. for 10 min. at 2000 g in an angle centrifuge to remove large particles. A second cycle of high and low speed centrifugation was performed on the supernatant solution.

The final virus suspension was a white opalescent liquid estimated by infectivity test to contain 55-70% of the virus in the original allantoic fluid. Such suspensions contained about 2.5 mgm. of virus per ml., calculated from phosphorus analyses, assuming that the virus contained 0.97% P (14).

In some cases the above procedure was modified in that the infected eggs were chilled at 5° C. for 18 hr. before harvesting, and washed chicken erythrocytes were added to the clear allantoic fluid to make a 2% suspension. This method was not as rapid as the one outlined above and suffered the further disadvantage that it was more difficult to maintain sterile conditions. Various media, such as the Ringer – calcium chloride solution described by Taylor *et al.* (15), and phosphate buffer, were used to resuspend the sedimented virus but offered no advantage over 0.85% sodium chloride solution.

Mounts were prepared from several of the purified virus suspensions, fixed in osmic acid vapor, and photographed in the electron microscope before and after shadow casting with chromium.* In every case the great majority of the particles observed were typical in size and shape of influenza virus with little other electron absorbing material present. In some cases a few of the filamentous forms often observed previously in preparations of this virus were apparent, see for example (13).

Freshly purified suspensions of virus gave a single boundary in the ultracentrifuge, Fig. 1, with a sedimentation constant of about 670×10^{-13} (uncorrected) in agreement with earlier work on the elementary bodies of influenza virus A. With some preparations a slightly raised base line ahead of the main component in sedimentation pictures suggested a small amount of faster moving material, probably clumped virus particles. When purified preparations were allowed to stand several days at 5° C. a small slower moving boundary appeared as described by Friedewald and Pickels (3). The sedimentation velocity measurements were carried out in a Spinco electrically driven ultracentrifuge equipped with a Philpot-Svensson type of optical

* *We are much indebted to Dr. G. D. Scott of the Physics Department, University of Toronto, for making the electron micrographs.*

PLATE I

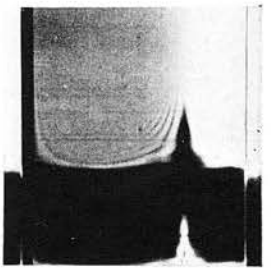

FIG. 1. *Refractive index photograph taken after eight minutes at 13,410 r.p.m. in the ultracentrifuge showing sedimenting boundary of influenza virus A in 0.85% sodium chloride solution at pH 7.0. Direction of sedimentation from right to left. Concentration of virus solution 2 mgm. per ml.*

system. A description of the centrifuge has been given by Rhodes and van Rooyen (13).

In eight different preparations of purified virus one ID_{50} for embryonated eggs contained $10^{-14.3}$ to $10^{-15.4}$ gm. of nitrogen.* For five of these preparations the results fell between the limits $10^{-14.85}$ to $10^{-15.10}$ gm. nitrogen per ID_{50}. The influenza virus particle was computed to have a weight of approximately $10^{-15.2}$ gm. assuming a spherical shape of diameter 100 mμ and a density of 1.22 gm. per cc. Since Taylor (14) has found the virus to contain 10.0% nitrogen it was calculated from the above infectivity figures that the purified preparations contained, on the average, about 16 virus particles in one ID_{50}. Friedewald and Pickels (3) determined about 10 particles per ID_{50} in their purified preparations of PR8 virus.

Incorporation of Radioactive Phosphorus into Influenza Virus

In the experiments on the incorporation of radioactive phosphorus into the virus, P^{32}, as inorganic phosphate, was diluted to the desired activity in sterile 0.85% saline adjusted to pH 7 with sodium hydroxide. Unless stated otherwise, 0.2 ml. of the solution of P^{32} was injected into the allantoic cavity of the embryonated egg at an arbitrarily chosen interval three hours following inoculation of influenza virus by the same route. After 48 hr. incubation at 36° C., the allantoic fluid was harvested and the purification of the virus followed the general procedure already given.

Having ascertained that under these conditions the purified virus suspensions contained measurable amounts of P^{32}, it was necessary to ensure that the P^{32} was, in fact, closely associated with the virus and could not be removed by repeated washing of the elementary bodies. The following experiment was designed to settle this point. Total phosphorus and P^{32} estimations were carried out on a number of the fractions obtained during the preparation of the purified radioactive virus and are reported in Table I. The specific activity is defined as the ratio of P^{32} in c.p.m. to total phosphorus in μgm.

Two hundred and nine 11-day embryonated eggs were inoculated with influenza virus. Three hours later each egg received 91,200 c.p.m. of P^{32}, contained in 0.2 ml. of 0.85% saline. During the ensuing 48 hr. incubation period 61 embryos died and were discarded. The remaining eggs were harvested and the allantoic fluid was allowed to stand overnight at 5° C. The agglutinated cells were removed by centrifugation and the supernatant solution (Supernatant A) was discarded. After washing the cells with Ringer – calcium chloride solution, the wash liquid (Supernatant B) was discarded. Fresh Ringer – calcium chloride solution was added and the virus eluted from the cells which were then removed by centrifugation and discarded. This virus solution was centrifuged at 20,000 g for one hour (Supernatant C), and the pellet resuspended in Ringer – calcium chloride and centrifuged at

* *These figures were given in error in the preliminary paper as* 4×10^{-15} *to* 5×10^{-14} *gm. nitrogen; they should have read* 4×10^{-16} *to* 5×10^{-15} *gm. nitrogen.*

TABLE I

SPECIFIC ACTIVITY OF RADIOACTIVE VIRUS DURING PURIFICATION PROCEDURE

Fraction	Volume of fraction, ml.	Total P^{32} c.p.m.	Specific activity of virus, c.p.m./μgm. P
Supernatant A	1010	6,868,000	—
Supernatant B	101	47,000	—
Supernatant C	95	26,000	—
Virus suspension 1	9.5	3850	15.2
Supernatant D	7.6	225	—
Virus suspension 2	7.6	2340	15.0
Supernatant E	5.5	32	—
Virus suspension 3	5.5	1465	16.6
Supernatant F	3.5	Trace	—
Supernatant G	3.5	Trace	—
Supernatant H	3.5	Trace	—
Virus suspension 6	3.5	612	15.9

2000 g for 10 min. to remove large particles and agglutinated virus. The resulting supernatant solution (Virus suspension 1) was subjected to two similar cyles of high and low speed centrifugation to give Supernatants D, E, and Virus suspensions 2, 3, the third virus suspension being made in 0.85% saline. Supernatant F was obtained by centrifuging this suspension at 20,000 g, the virus pellet was resuspended in saline containing 0.01 *M* phosphate buffer, pH 7.0, and allowed to stand 48 hr. at 5° C. to permit any exchange of the P^{32} of the virus with the buffer phosphate. Following centrifugation at 20,000 (Supernatant G) the virus was resuspended in saline, sedimented at high speed (Supernatant H), resuspended in saline, and finally centrifuged for 10 min. at 2000 g to remove the larger particles. The supernatant solution was Virus suspension 6 shown in Table I.

It can be seen from Table I that despite the repeated washings received by the elementary bodies and the opportunity allowed for exchange of P^{32} to occur with phosphate buffer, the specific activities of the various virus suspensions remained essentially constant. This finding indicated that the P^{32} was firmly fixed in the virus. Similar results were obtained in three further such experiments.

Control Experiments on Addition of Radioactive Phosphorus to Infectious Allantoic Fluid

The previous experiment demonstrated that influenza virus grown in the embryonated egg in the presence of P^{32} contained a definite amount of the isotope. There was the possibility, however, that the isotope had not been incorporated into the virus during its actual growth in the cell, but after the virus had been liberated from the cells of the membrane into the allantoic fluid. As described later, a considerable amount of the injected P^{32} remained

in the allantoic fluid, even at the end of the 48 hr. incubation period. Since maximum growth of the virus is almost complete in the first 24 hr. after infection (6), a considerable proportion of the freshly liberated virus would remain in contact with the radioactive allantoic fluid under conditions favorable to exchange of the isotope with the virus phosphorus. To gain information on this point an experiment was carried out in which P^{32} was added to freshly harvested infectious allantoic fluid from which the virus was subsequently isolated and its isotope content determined.

Allantoic fluid was collected from 97 embryonated eggs which had been infected with influenza virus two days previously and allowed to incubate in the usual way. Care was taken to exclude red blood cells when harvesting the fluid. Radioactive phosphate was added to the fluid to give 75,000 c.p.m. per ml., an amount corresponding to that used in the previous experiments on labelling the virus. After standing two days at 5° C. to allow any exchange to take place, chicken red cells were added to make a final 2% suspension. The virus suspension, obtained in the usual way by elution from the cells into saline, was subjected to four cycles of differential centrifugation, the resuspended virus after each step being analyzed for total P and P^{32} as shown in Table II.

TABLE II

SPECIFIC ACTIVITY OF VIRUS AFTER ADDITION OF P^{32} TO INFECTIOUS ALLANTOIC FLUID

Fraction	Volume of fraction, ml.	Total P^{32}, c.p.m.	Specific activity of virus, c.p.m./μgm. P
Infectious allantoic fluid	471	35,200,000	—
Supernatant from agglutinated red cells	460	32,800,000	—
Wash liquid from agglutinated red cells	46	742,000	—
Supernatant 1 after 20,000 g	45.5	120,900	—
Virus suspension 1	13.6	374	2.9
Supernatant 2 after 20,000 g	11.6	276	—
Virus suspension 2	11.6	24	0.3
Supernatant 3 after 20,000 g	9.6	0	—
Virus suspension 3	9.6	48	0.7
Supernatant 4 after 20,000 g	7.6	0	—
Virus suspension 4	7.6	0	—

It is seen that the virus suspension contained a negligible amount of radioactivity as evidenced by its specific activity of 0.3 c.p.m./μgm. P. Such small amounts of radioactivity as were contained in this suspension, where the counting rate was two to three counts above background, were difficult to estimate with any accuracy. It is apparent, however, that little or no exchange had taken place between the virus and inorganic radioactive phosphate. Three further such experiments yielded similar results.

Control Experiments on Addition of Radioactive Phosphorus to Purified Influenza Virus

Although the previous control experiments indicated that influenza virus in infectious allantoic fluid did not take up P^{32} *in vitro* from radioactive phosphate, it was thought of interest to add relatively large amounts of P^{32} to a purified virus suspension to determine the efficiency of the differential centrifugation procedure in removing the isotope.

A suspension of influenza virus in saline was prepared in the usual way with two cycles of differential centrifugation. Radioactive phosphate was added to the purified virus to give a final concentration of 69,600 c.p.m. per ml. of suspension. After standing 24 hr. at 5° C. to permit any exchange, or adsorption of P^{32} on the virus particles, the virus was sedimented at 20,000 g. The supernatant was decanted (Supernatant 1), a small quantity of saline was added to wash down the tube and quickly poured off, and the pellet resuspended in saline. This procedure was repeated three times; total phosphorus and P^{32} estimations were carried out on the various fractions, the results being shown in Table III.

TABLE III

SPECIFIC ACTIVITY OF VIRUS AFTER ADDITION OF P^{32} TO PURIFIED SUSPENSION

Fraction	Volume of fraction, ml.	Total P^{32}, c.p.m.	Specific activity of virus, c.p.m./μgm. P
Virus suspension with added P^{32}	4.6	320,100	—
Supernatant 1 after 20,000 g	4.6	288,500	—
Virus suspension 1	13.8	3160	23.5
Supernatant 2 after 20,000 g	13.8	2900	—
Virus suspension 2	11.8	73	0.74
Supernatant 3 after 20,000 g	11.8	Trace	—
Virus suspension 3	8.5	83	0.89
Virus suspension 4	6.5	51	0.79

From the specific activity figures it is apparent that little of the added P^{32} remained in the second virus suspension. A small amount of radioactivity remained in the virus and was difficult to remove, but too much confidence cannot be placed in the radioactivity estimations for the second, third, and fourth virus suspensions because of the very low counting rate. A further such experiment gave similar results.

Specific Activity of Influenza Virus Grown in Presence of Different Amounts of Radioactive Phosphorus

Fig. 2 shows the variation in specific activity of influenza virus when grown in the embryonated egg in the presence of different amounts of P^{32}. One experiment was carried out to illustrate this point. Two groups of 11-day embryonated eggs were inoculated with influenza virus then with P^{32} after

three hours, 14,850 c.p.m. in each egg in the first group and 64,500 c.p.m. in the second. The remaining data were gathered incidentally from eight other experiments performed during the course of the work. In every case the

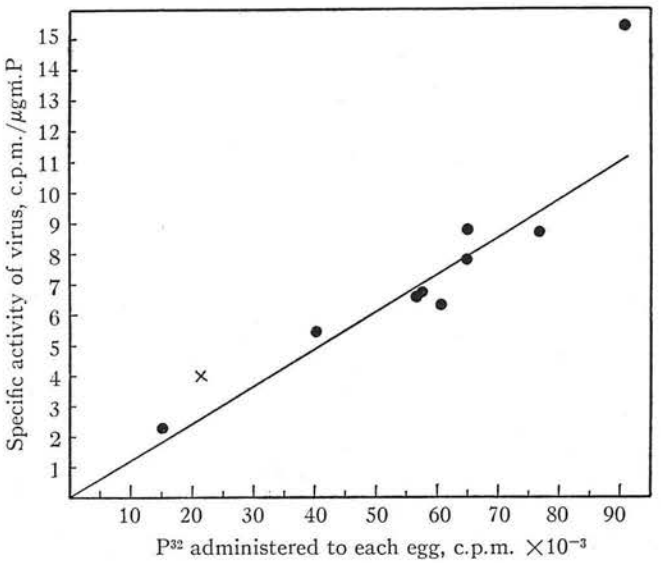

FIG. 2. *Relationship of specific activity of purified radioactive virus to amount of radioactive phosphorus administered.*

specific activity of the virus was determined after purification in the usual way with two cycles of differential centrifugation, except for one preparation which received only one cycle of centrifugation. The latter preparation is represented by a cross in the figure.

It is seen that the specific activity of the virus rises in a linear manner with the amount of injected P^{32} up to at least 91,200 c.p.m. in each egg.

Effect of β-Radiation from Radioactive Phosphorus on the Embryo and on the Growth and Properties of Influenza Virus

During the course of the work it was found that the death rate of embryonated eggs injected with influenza virus A by the allantoic route might be as high as 6% during the succeeding 48 hr. incubation period and on the average was about 3-4%. When radioactive phosphorus was injected also, by the allantoic route, the embryo death rate increased until, with 70,000 c.p.m. injected into each egg, 10 to 30% of the embryos died; there was a large variation in the number of embryo deaths from one experiment to another. It seems probable that many of these deaths resulted from the effect of β-radiation, the dead embryos often appearing markedly haemorrhagic. This finding put a practical upper limit on the amount of isotope which could be injected into each egg and, consequently, an upper limit on the amount of isotope which could be incorporated into the influenza virus.

In spite of the fact that, in several experiments, many of the embryos died from radiation injury, the yield of influenza virus from the surviving eggs was no less than from eggs which had not received P^{32}. This was confirmed on several occasions by doing infectivity titrations and CCA tests on the pooled allantoic fluid from eggs which had survived the injection of up to 150,000 c.p.m. of P^{32}.

In addition, the properties of purified influenza virus containing radioactive phosphorus, up to a specific activity of 15 c.p.m./μgm. P did not seem to be measurably different from those of normal virus. The appearance in electron micrographs and the sedimentation constant were unchanged. Infectivity figures given in an earlier section for eight different preparations of purified virus include three radioactive preparations; there was no significant difference between the radioactive and normal virus preparations.

It would appear, therefore, that relatively severe irradiation of the allantoic membrane, where influenza virus multiplication is presumed to occur, did not interfere appreciably with the virus growth, nor did it alter the properties of the purified virus.

Specific Activity of Influenza Virus with Varying Intervals Between Virus and Radioactive Phosphorus Inoculation into Embryonated Eggs

Previous experience had indicated that the maximum rate of growth of the PR8 strain of influenza virus A occurred during 24 hr. after inoculation of the egg. It was assumed that if P^{32} were injected into the allantoic cavity while the virus was rapidly growing, conditions should be favorable for incorporation of the isotope into the virus, since the cells of the allantoic membrane should be in intimate contact with P^{32} during this period. Consequently, in all experiments involving labelling of influenza virus with P^{32} so far reported in this paper, radioactive phosphorus was injected into embryonated eggs three hours following inoculation of virus.

There was a possibility, however, that P^{32} uptake by the growing virus might be much greater if some of the normal phosphorus containing constituents in the membrane cells were labelled through injection of the isotope at some time prior to infection with influenza virus. Therefore, a single experiment was carried out in which a group of 50 embryonated eggs was inoculated with P^{32} at each of the intervals, 48 hr. before, 24 hr. before, a few minutes after, and 24 hr. after infection with 10-100 ID_{50} of influenza virus. Each egg of the four groups received 76,000 c.p.m. of the isotope. After incubation of the eggs the virus from each group was purified, as usual, analyzed for P^{32} and total phosphorus, and the specific activity was calculated. The results are shown in Table IV.

It would appear that when P^{32} was injected into eggs 48 hr. previous to virus infection, the specific activity of the virus was about 22% higher than when the isotope was administered almost simultaneously with the virus. When the isotope was injected 24 hr. after infection the specific activity of the virus was very low, and it is worth noting that this finding supports

strongly the previous indication that little or no exchange occurs between virus and P^{32}. In this case the virus, freshly liberated from the allantoic membrane during the 24 hr. following infection, remained in contact, *in vivo*,

TABLE IV

SPECIFIC ACTIVITY OF INFLUENZA VIRUS WHEN P^{32} INJECTED INTO EMBRYONATED EGGS AT DIFFERENT TIMES WITH RESPECT TO TIME OF VIRUS INOCULATION

Time of injection of P^{32}	Specific activity of purified virus c.p.m./μgm. P
48 hr. before virus	10.5
24 hr. before virus	9.8
Same time as virus	8.6
24 hr. after virus	0.56

with allantoic fluid containing large amounts of P^{32} for a further 24 hr. The low specific activity of the virus from this group of eggs, compared to the very much higher specific activities obtained in the other three groups, indicates that the virus is labelled with the isotope only during its growth in the cells of the membranes. Even the low specific activity observed in the fourth group could be accounted for by the relatively small amount of virus multiplication which occurred during the second 24 hr. period following infection of the eggs.

Disappearance of Radioactive Phosphorus from Allantoic Fluid and from Yolk Sac of Embryonated Egg

During the course of the work it was felt necessary to obtain information on the amount of P^{32} remaining in the allantoic fluid at various intervals following injection of P^{32}. Experiments designed for this purpose were carried out as follows:

Each of a number of 11-day embryonated eggs was injected with 0.2 ml. of P^{32} solution by the allantoic route, the isotope being dissolved either in saline or in 0.1 *M* phosphate buffer, pH 7. Within 5 to 10 min. after injection the allantoic fluid of five eggs was harvested and the total volume of fluid measured. Radioactivity estimations were carried out on the combined fluids. Control experiments, carried out by diluting a saline solution of known P^{32} content in normal allantoic fluid and measuring the activities of various dilutions, indicated that the presence of allantoic fluid did not interfere with the method of assay. The remaining eggs were incubated at 36° C. and, at intervals, groups of five eggs each were treated in the same manner as above.

In all, 10 such experiments were performed, the amount of P^{32} injected per egg varying between 8700 c.p.m. to 40,900 c.p.m. from one experiment to

another. The results of two of the experiments, which are representative of the remainder, are shown in Fig. 3.

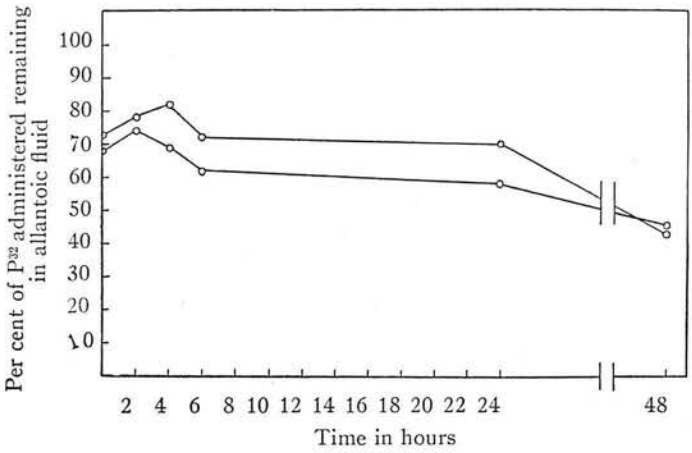

FIG. 3. *Rate of disappearance of P^{32} from the allantoic fluid of 11-day embryonated eggs.*

In none of the experiments was more than 90% of the administered P^{32} recovered from the groups of eggs harvested at zero time. This was probably partly due to the fact that it was impossible to harvest the allantoic fluid quantitatively. The slight increase in P^{32} recovered in the allantoic fluid, shown between two to four hours in Fig. 3, was observed in each of the remaining eight experiments, although in some cases the increase was not seen until four to six hours after injection of isotope. These findings were not considered of immediate importance and were not investigated further. In two experiments, 42% and 51% of the administered isotope was recovered after 72 hr.

Although similar experiments were not done with influenza virus infected eggs, it was observed incidentally during another part of the work that 40% to 50% of the administered P^{32} was recovered after 48 hr. from the allantoic fluid of infected eggs.

Two experiments were carried out to determine whether P^{32} injected into the yolk sac of 11-day embryonated eggs would appear rapidly in the allantoic fluid. Since the technique of inoculation into the yolk sac is not easy it was necessary to ensure that the isotope was placed in the desired position. Consequently the P^{32} solution, 46,000 c.p.m. into each egg, was made up in 2% trypan red solution for inoculation. It had been determined previously that trypan red remained indefinitely in the yolk sac and that it was innocuous to the developing embryo. On harvesting the eggs the allantoic fluid was used only from those eggs in which the dye was localized in the yolk sac.

After 72 hr., only 1.5% of the administered P^{32} had appeared in the allantoic fluid.

Discussion

It is considered that the suspensions of purified virus handled throughout this work consisted almost entirely of the elementary bodies of influenza virus. Sedimentation (3, 15), electron microscope (15, 17), infectivity results (3), and chemical analysis (5, 14) were in accord with the observations of previous workers and together suggested that little or no soluble or particulate matter other than virus was present in these preparations.

It would appear that the P^{32} in the radioactive virus was, in fact, incorporated into the structure of the virus. This is indicated by the findings that virus does not exchange with P^{32} and that the isotope in labelled virus cannot be removed by repeated washings of the elementary bodies. Further evidence was offered by the observation, made during chemical analysis of radioactive virus, that both the phospholipid and nucleic acid fractions of purified virus contained P^{32} (5).

In radioactive tracer experiments with biological materials, it is essential that the radiation from the isotope should not interfere with the metabolism of the system under observation. In such studies, it is generally easy to exclude radiation effects since only minute amounts of tracer are required. However, for the purpose of further study of the labelled influenza virus, it was necessary to obtain virus with high specific radioactivity. It became apparent early in the work that in order to achieve this end, relatively large amounts of P^{32} would have to be injected into the virus infected egg. In many experiments, when 450-600 μrd. of P^{32} or more were injected into each egg, a significant proportion of the embryos died.

It was considered that these deaths may have resulted from some toxic material present in the P^{32}. However, the isotope solutions, as received from the National Research Council, were usually diluted at least one thousandfold before administration to the eggs in 0.2 ml. amounts. It was thought improbable that any compound would be present in sufficiently high concentration in the original solution to cause the high mortality observed in some of the groups of fertile eggs. It was therefore presumed that death of the embryos was in large measure due to β-radiation injury.

In spite of these observations it is noteworthy that in the infected eggs which survived the injection of as much as 1300 μrd. of P^{32} the yield of virus was not appreciably less than from eggs which had not received the isotope. It was noted also that the specific activity of purified radioactive virus rose linearly with increasing amounts of P^{32} administered to the infected eggs up to at least 775 μrd. for each egg. If radiation were interfering with the growth of virus both the yield and specific activity of the virus might be expected to decrease rapidly with increasing amounts of isotope administered. It would thus appear that the mechanisms involved in the growth of influenza virus are relatively resistant to β-radiation.

One of the main points we had hoped to investigate with the radioactive virus was the fate of the isotope when the virus was growing in the allantoic

membrane. So far such a study has been precluded by the small amount of P^{32} which has been introduced into the virus. It can be calculated that the specific activity of the radioactive virus would have to be increased at least a thousandfold over the highest value yet obtained in order to study this problem to any advantage. While there would seem to be little hope of obtaining influenza virus with such a high specific activity by the methods reported here, it is a subject for investigation whether the activity might be increased substantially by administering the isotope by some route other than by the allantoic sac.

The infectivity, sedimentation characteristics, and morphology of the purified radioactive virus were not significantly different from those of unlabelled virus. It was calculated that, for influenza virus having a specific activity of 1.29 μrd. per μgm. P (15 c.p.m. per μgm. P), the proportion of radioactive phosphorus atoms to total phosphorus atoms in the virus was 1 to 8.4×10^9. That is, on the average, one radioactive phosphorus atom was present for every 66,500 virus particles. Therefore, even if the properties of labelled virus were markedly different from those of unlabelled virus, the presence of such a small proportion of radioactive virus particles would hardly be expected to cause a measurable change in the biological or physical properties of the virus preparation. Thus it is not possible to state as yet whether labelled virus behaves in the same manner as unmarked virus.

Acknowledgments

This work has been aided by a grant from the National Cancer Institute of Canada. We are indebted to Miss E. K. Campbell for technical assistance and to Dr. C. E. van Rooyen and Dr. A. J. Rhodes for their interest throughout.

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THE CHEMICAL ANALYSIS OF PURIFIED INFLUENZA VIRUS A (PR8 STRAIN) CONTAINING RADIOACTIVE PHOSPHORUS

By A. F. Graham

Reprinted from Canadian Journal of Research, E, 28 : 186-195. October, 1950

THE CHEMICAL ANALYSIS OF PURIFIED INFLUENZA VIRUS A (PR8 STRAIN) CONTAINING RADIOACTIVE PHOSPHORUS[1]

By A. F. Graham

Abstract

Purified influenza virus A (PR8 strain) was found to contain about 11% phospholipid and 5% nucleic acid in agreement with previously reported work. The method of Schmidt and Thannhauser, applied to the nucleic acid fraction of the virus, indicated the presence of 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid. When influenza virus was grown in the allantoic membrane of the embryonated egg in the presence of inorganic radioactive phosphorus both phospholipid and nucleic acid components of the virus were found to contain the isotope. The specific radioactivity of the nucleic acid fraction was about four times that of the phospholipid.

Introduction

In previous work (4, 5) it was shown that when the PR8 strain of influenza virus A was grown in the allantoic membrane of the embryonated egg in the presence of inorganic radioactive phosphate, the virus, when subsequently purified, contained a small amount of the isotope. There was no exchange of P^{32} between the virus and inorganic phosphate and it was concluded that the virus had been labelled with the isotope during its growth in the cells.

Since it has been reported by Taylor (15) that the greater part of the phosphorus of the virus is contained in the phospholipid and nucleic acid it was of interest to determine whether the P^{32} in the radioactive virus was also present in these components. If either or both of the phospholipid and nucleic acid were found to be labelled, it would afford additional evidence that the isotope was incorporated into the structure of the virus. The work described in this paper indicates that both phospholipid and nucleic acid were labelled.

Analyses of the nucleic acid content of influenza virus, using quantitive colorimetric tests for pentose and desoxypentose, have been reported by Taylor (15) and by Knight (7). While both pentose and desoxypentose nucleic acids seem to be present, there is still some doubt over their exact proportions, as pointed out by Beard (1). The present work indicates that these colorimetric tests for nucleic acids may be subject to interference from other constituents present in the virus particle and consequently an independent method, that of Schmidt and Thannhauser (11), has been applied in an attempt to determine the relative proportions of the two nucleic acids. From the results of these analyses it would appear that the PR8 strain of influenza virus A contains about 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid.

[1] *Manuscript received June 6, 1950.*

Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. Aided by a grant from the National Cancer Institute of Canada.

Methods

Determination of Total Phosphorus

The method has been fully described in a previous paper (5).

Determination of Radioactive Phosphorus

All the radioactivity measurements were made on solutions of material in water or organic solvents. The method, which was also used throughout the previously reported work (4, 5), is described here in detail.

The radioactive solutions were pipetted onto $1\frac{1}{8}$ in. diameter filter paper discs fixed to the tacky surface of a 2 in. wide strip of "Scotch" cellophane tape. After drying the discs under an infrared lamp a second strip of tape was pressed firmly over the top. An aliquot of 0.2 ml. of solution was sufficient to saturate the paper discs; where the radioactivity of the material was low, several 0.2 ml. aliquots were added to each disc, the paper being dried between additions. The cellophane tape was trimmed off close to the edge of the disc, leaving a "sandwich" of filter paper between two layers of tape. This was fixed in a brass mount and inserted under a Geiger-Müller counter tube of the end-window type. The brass mount was constructed in such a way that the radiation from an area of the filter paper exactly 1 in. in diameter registered on the counter.

A routine set of five radioactivity determinations was made with this method on an aqueous solution of P^{32}, as inorganic phosphate, to determine its accuracy. With an average counting rate of 3450 impulses per min. (150 times background) the standard error of the mean was 1.1%. Inverting the paper discs under the counter had no measurable effect on the counting rate, nor did a total of three thicknesses of cellophane tape over the discs reduce the number of impulses registered.

As the method was used for estimating the radioactivity in biological fluids which contained appreciable amounts of protein, it was necessary to determine whether the presence of protein would interfere with the determination. Accordingly, a series of twofold dilutions of a solution of P^{32}, as inorganic phosphate, was made in 2% haemoglobin. At the same time, a similar series of dilutions of the same solution was made in distilled water. Radioactivity determinations were made on each series of dilutions as described above. In both series, the activity decreased in direct ratio to the concentration of P^{32}, the activities of corresponding dilutions in each series being the same within experimental error.

The method was calibrated against aqueous solutions of two uranium salts (C.P. grade) both of which had stood at least two years to ensure that they were in equilibrium. The β-ray disintegration rates of the uranium sources were calculated from data given by Kamen (6). This calibration was later checked against a RaD + E standard source from the National Bureau of Standards, Washington. A solution of P^{32} calibrated against the Washington

standard contained 5330 μrd. per ml. When measured against a uranium acetate solution it contained 5880 μrd. per ml. and against a uranium nitrate solution 5950 μrd. per ml.

In this paper, as in the previous report (5), the activities of P^{32} sources are expressed in counts per minute (c.p.m.); the results may be converted to microrutherfords using the factor 1000 c.p.m. as equivalent to a β-ray activity of 85.8 μrd.

Determination of Pentose Nucleic Acid (PNA) by the Orcinol Method

PNA was estimated by the orcinol method according to the directions of McRary and Slattery (10). Two specimens of commercial yeast nucleic acid containing 7.90% P (Schwartz) and 7.85% P (Eimer and Amend) were used as standards. Although the green color developed in this test is due to the pentose in PNA the results were expressed in terms of the phosphorus content of the nucleic acid, that is, as pentose nucleic acid phosphorus (PNAP). When per cent transmittance, read at 660 mμ against reagent blanks, was plotted against PNAP for the two PNA specimens the curves were linear between 0-13 μgm. PNAP and superimposable.

According to Schneider (12, 13) the nucleic acids of animal tissue may be completely extracted with 5% trichloroacetic acid (TCA) at 95° C. This method was applied to one of the specimens of PNA (Schwartz). The nucleic acid was extracted completely in 15 min., judged by phosphorus analysis, leaving a small residue presumably of protein. When the orcinol reaction was applied to a series of dilutions of the extract and the results plotted in terms of phosphorus content, the curve was identical with the two former curves.

Since the slopes of standard curves obtained in this test varied from one set of determinations to another owing to small variations in conditions of color development, a set of five dilutions of one of the standard PNA solutions was always run at the same time as an unknown solution. Thus the color density of the unknown solution could always be referred to a standard curve obtained under exactly the same conditions.

Determination of Desoxypentose Nucleic Acid (DNA) by the Diphenylamine Reaction

The diphenylamine reaction for DNA was carried out according to the directions of Seibert (14). Two specimens of calf thymus nucleic acid containing 7.32% P and 8.20% P were used as standards.* When per cent transmittance at 600 mμ of the color developed in the diphenylamine reaction was plotted against phosphorus content for each of the two specimens, the two curves were superimposable and linear between 0-50 μgm. DNAP. A weighed quantity of one of the specimens was extracted with 5% TCA at 95° C. The extract contained all the phosphorus of the original DNA and in the diphenylamine test gave a curve almost identical to the two former curves.

* *I am indebted to Dr. G. C. Butler for one of the specimens of calf thymus nucleic acid.*

As in the orcinol test a set of five dilutions of a standard DNA solution was always run simultaneously with an unknown solution.

Preparation of Purified Influenza Virus

Purified influenza virus was prepared by the method which has already been fully described (5).

Experimental

Chemical Analysis of Influenza Virus

Before carrying out any analytical work on radioactive virus, it was necessary to determine the distribution of phosphorus between the virus constituents. For analysis, suspensions of purified influenza virus were prepared. An aliquot was taken for total phosphorus estimation and a further aliquot for dialysis against several changes of distilled water at 5° C. After 48 hr. the agglutinated virus was sedimented, dried *in vacuo* from the frozen state and then over phosphorus pentoxide in a vacuum desiccator.

The weighed dry material (10-25 mgm.) was stirred mechanically for 45 min. at room temperature with 4 ml. of alcohol–ether mixture (3/1, v./v.). The extraction was repeated on the sediment after centrifugation and the material was finally washed once with a small amount of ether and dried *in vacuo*. This alcohol–ether insoluble residue was considered to contain the protein and nucleic acid of the virus.

The combined alcohol–ether extracts were evaporated to dryness at 30° C. in a stream of nitrogen and the residue was extracted with several small portions of petroleum ether (b.p. 40°-60° C.) at room temperature. In accordance with the earlier work of Taylor (15) this extract was assumed to contain the phospholipid of the virus. Phosphorus and weight determinations were made on the various fractions.

The results of the fractionation are shown in Table I, the figures representing analyses on four different virus preparations. In each row of the table, the figures are averages of results obtained on at least three of the four preparations since all the determinations were not carried out on every preparation.

TABLE I

Chemical analysis of influenza virus A (PR8 strain)

Fraction	Percentage of virus weight*	Phosphorus, % of virus weight*
Dried whole virus	100	0.96
Alcohol–ether soluble	26	0.51
Petroleum ether soluble	24	0.48
Petroleum ether insoluble	—	0.04
Alcohol–ether insoluble	76	0.45

* *Values are based on dry weight of virus.*

The virus would appear to contain about 11% phospholipid, calculated from the phosphorus content of the petroleum ether soluble fraction. The results are in agreement with those of Taylor (15) for this virus.

Fractionation of the Alcohol–Ether Insoluble Residue

A strongly positive orcinol test for pentose was obtained with the alcohol-ether insoluble residue. While an unmistakably positive diphenylamine test for desoxypentose was not obtained on this fraction nor on whole virus, in our hands this has proved to be a relatively insensitive color reaction. Moreover the diphenylamine reaction is subject to interference by proteins and other substances as indicated by von Euler and Hahn (17).

Therefore, an attempt was made to separate and estimate the two nucleic acids of the virus by the method of Schmidt and Thannhauser (11). This procedure was tested out on a mixture of calf thymus nucleic acid, yeast nucleic acid, and crystalline egg albumen of known phosphorus contents, the recovery of the two nucleic acids being almost quantitative when present in roughly equal proportions. When the method was applied to the alcohol-ether insoluble residues of four different preparations of influenza virus 94.0%, 90.6%, 93.7%, and 93.5% of the total phosphorus of the residue appeared with the PNA fraction.

If phosphoprotein were present in the virus, its phosphorus would appear in the PNA fraction as inorganic phosphate. The procedure of Delory (3) was therefore applied to the separated PNA fraction, in two cases, to precipitate any inorganic phosphorus so that it could be estimated separately. In neither case was more than a trace of inorganic phosphorus found. It is well to mention, however, that the precipitation of inorganic phosphate was not quantitative when the amount of inorganic phosphorus present was less than 15 μgm. per ml. To avoid this difficulty the precipitation method of Delory was applied to solutions containing known amounts of inorganic phosphate (0-30 μgm. P per ml.) and a calibration curve prepared relating inorganic P added to that recovered from the precipitation. When a solution was made up containing 8.70 μgm. P per ml. as inorganic phosphate and 5.55 μgm. P per ml. as yeast nucleic acid phosphorus, using the precipitation method and calibration curve, the inorganic phosphate was estimated as 8.40 μgm. P per ml.; the precipitation was carried out under the same conditions as obtained in the influenza virus experiments.

It would appear from these results that about 6% of the phosphorus of the alcohol–ether insoluble residue of the virus is present in DNA and about 94% in PNA. This would be equivalent to approximately 0.3% DNA and 4.5% PNA in the whole virus.

Determination of PNA and DNA in the Virus by Colorimetric Tests

Since the above results for PNA and DNA in the virus were quite different from previously reported results (7, 15) an attempt was made to determine the two nucleic acids by quantitative colorimetric tests.

Following separation of the two fractions from the alcohol–ether insoluble residue of the virus by the Schmidt and Thannhauser method, both orcinol and diphenylamine tests were carried out on the PNA fraction. It was observed that practically all the phosphorus could be extracted from the DNA fraction with 5% trichloroacetic acid for 30 min. at 90° C.; the diphenylamine test for DNA was carried out on this extract. The results are recorded in Table II. Since the orcinol and diphenylamine reactions were standardized

TABLE II

DISTRIBUTION OF PHOSPHORUS IN COMPONENTS OF ALCOHOL-ETHER INSOLUBLE RESIDUE OF INFLUENZA VIRUS SEPARATED BY SCHMIDT AND THANNHAUSER METHOD

Fraction	Total phosphorus, μgm.	Phosphorus by orcinol test, μgm.	Phosphorus by diphenylamine test, μgm.
Pentose nucleic acid	62.8	53.8	7.7
Desoxypentose nucleic acid	4.3	—	2.6

in terms of the phosphorus contents of the respective nucleic acids as explained earlier, the figures in columns three and four of Table II give the amounts of phosphorus associated with these two acids in the virus. It is observed that the total amount of phosphorus determined from the colorimetric tests in this experiment checks fairly well with the total phosphorus estimated by direct determination. The PNA fraction separated by the Schmidt and Thannhauser method would appear to contain a small amount of DNA judged by the results of the diphenylamine test. The orcinol reaction was carried out on the DNA fraction but it was impossible to determine whether or not a slight green color had formed because of the presence of a brown pigment which appeared during color development. Similar results were obtained with a second preparation of virus.

It should be remarked that despite the reasonable agreement between the results of the two methods as shown in Table II, the colorimetric estimations came under suspicion for two reasons. Firstly, both ribose and yeast nucleic acid yielded a clear brilliant green color in the orcinol reaction whereas the influenza virus fractions often gave an olive green color with a tendency to slight opalescence. Secondly, the diphenylamine tests on the virus fractions were almost invariably slightly brown in color instead of the pure blue obtained with DNA in this reaction. Both these observations suggested the presence of substances in the virus which might interfere in the color tests.

Evidence that the colorimetric tests were not entirely trustworthy when applied to influenza virus fractions was obtained in a further experiment. The alcohol–ether insoluble residue from a purified virus preparation was treated for 30 min. with 5% trichloroacetic acid at 90° C. in an attempt to extract completely the nucleic acids. Eighty-eight per cent of the phosphorus

was removed by this treatment, 87% in a second such experiment. Both orcinol and diphenylamine tests were applied to the extract and to the extracted residue. The results are shown in Table III. It is observed that

TABLE III

DISTRIBUTION OF PHOSPHORUS IN HOT TRICHLOROACETIC ACID EXTRACT AND RESIDUE OF ALCOHOL–ETHER INSOLUBLE PORTION OF INFLUENZA VIRUS

Fraction	Total P, μgm.	P by orcinol test, μgm.	P by diphenylamine test, μgm.
Trichloroacetic acid extract	167.3	157	41.8
Extracted residue	23.3	37.5	12.0

the total amount of phosphorus in the alcohol–ether insoluble fraction of the virus estimated by the colorimetric tests, was about 30% greater than the amount of phosphorus found to be present by the direct estimation of total phosphorus.

Application of the orcinol reaction of Tillmans and Phillipi (16), standardized against glucose, to the trichloroacetic acid extract mentioned above indicated a carbohydrate content of about 7% of the weight of the virus. Estimations of carbohydrate by this method were not done on the trichloroacetic acid extracted residue.

Chemical Fractionation of Radioactive Influenza Virus

In order to determine the distribution of P^{32} between the phosphorus containing constituents of the labelled virus, two samples of purified radioactive virus were prepared from two groups of eggs which for Preparation I received 56,700 c.p.m. of P^{32} in each egg, and for Preparation II, 65,000 c.p.m. in each egg. Following the adsorption and elution technique with red cells previously described, the virus was subjected to two cycles of differential centrifugation, the virus being resuspended each time in 0.85% (w./v.) sodium chloride solution.

A known volume of Preparation I was then dried *in vacuo* from the frozen state, while an aliquot of Preparation II was agglutinated by dialysis against distilled water before drying in the same manner. Both dried preparations were then fractionated by the same methods as described for the nonradioactive virus. Total phosphorus and P^{32} estimations were carried out on each fraction; the specific activities were calculated and are shown in Table IV along with the results for total phosphorus. The total phosphorus figures for whole virus represent the total amounts of phosphorus in the aliquots taken for drying and for dialysis in Preparations I and II respectively. Phosphorus and radioactivity estimations on the alcohol–ether insoluble fractions were

TABLE IV

SPECIFIC ACTIVITIES OF FRACTIONS OF INFLUENZA VIRUS LABELLED WITH P^{32}

Fraction	Preparation I		Preparation II	
	Total phosphorus, μgm.	Specific activity, c.p.m./μgm. P	Total phosphorus, μgm.	Specific activity, c.p.m./μgm. P
Whole virus	94.5	6.6	113.6	8.8
Petroleum ether soluble	46.0	3.0	17.9	3.6
Petroleum ether insoluble	1.5	Trace	33.5	3.6
Alcohol–ether insoluble	46.5	10.8	44.7	16.5

carried out on solutions of the material in 1.0 *N* potassium hydroxide, blank corrections being made for the very small natural radioactivity of potassium.

For Preparation II, the sum of the phosphorus figures for the three fractions is only 85% of the phosphorus in the whole virus. This difference could be accounted for by loss of virus in the manipulations attendant upon dialysis. Further, in this preparation the extraction of phospholipid from the alcohol–ether soluble fraction with petroleum ether seems to have been incomplete, judged by the amount of phosphorus containing material remaining in the petroleum ether insoluble fraction. The two experiments, however, are consistent in that they indicate the specific activity of the nucleic acid portion of the virus to have been 3.5 – 4.5 times that of the phospholipid.

In Preparation I, an attempt was made to purify the phospholipid by precipitation with magnesium chloride and acetone according to the method of Bloor (2) to find whether the specific activity would be changed by this treatment. After purification there was insufficient material left to allow an accurate radioactivity estimation. An attempt was also made to separate the PNA and DNA by the method of Schmidt and Thannhauser as described in a previous section. By far the largest proportion of the radioactivity was associated with the PNA fraction; it was impossible to get an accurate estimate of the small amount of activity remaining with the DNA.

Discussion

As far as the chemical analysis of purified influenza virus has been carried in the present work the results are in agreement with previous work, except for the relative amounts of pentose and desoxypentose nucleic acids. On the assumption that the phosphorus of the virus was contained only in phospholipid and nucleic acid, the total amount of nucleic acid was found to be about 5%, calculated from the phosphorus content of the lipid free fraction. It has been demonstrated by Knight (7) that both types of nucleic acid are present since a nucleic acid fraction separated from over 2 gm. of purified virus gave positive color reactions for both pentose and desoxypentose.

Knight concluded from his analyses that the virus contained about 2.3% pentose nucleic acid, later revised to 3.0% (8), based on the use of the orcinol color test for pentose. While no figures were given, Knight mentioned that there appeared to be 9 to 10 times more pentose than desoxypentose present. The initial work on the virus by Taylor (15) indicated the presence of 2.1% desoxypentose nucleic acid. Thus it would appear that there is not yet complete agreement on the analysis of the nucleic acid portion of the virus.

In order to separate the two nucleic acids of the radioactive virus to determine their isotope contents and also to estimate the two nucleic acids by an independent method, the technique of Schmidt and Thannhauser has been applied to the lipid free fraction of the virus. From the analyses it appeared that the virus contained about 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid. There was little or no phosphoprotein present. The same result was obtained with four different preparations of purified virus indicating, at least, that the technique of separation gave consistent results. Whether or not there was a clean separation of the two nucleic acids in the virus by this method cannot be deduced from phosphorus analyses alone. It has been demonstrated in the course of the present work that yeast and calf thymus nucleic acids can be separated almost quantitatively by the Schmidt and Thannhauser method, and similar results have been obtained with sheep liver pentose nucleic and calf thymus nucleic acids by McCarter and Steljes (9). However, it is not certain that the nucleic acids of influenza virus behave in the same manner as other nucleic acids when submitted to the action of dilute alkali.

In carrying out color tests on the separated nucleic acids of the virus in an attempt to confirm the above findings, it was observed that both the orcinol reaction for pentose and the diphenylamine reaction for desoxypentose might be subject to interference from other constituents of the virus. While the results of the colorimetric tests do not, therefore, give unqualified support for the estimations of the nucleic acids from phosphorus analyses of the separated acids, they are in agreement with the phosphorus analyses to the extent that they indicate the presence of much more pentose than desoxypentose nucleic acid.

The observation that in influenza virus containing P^{32} both phospholipid and nucleic acid fractions were labelled furnishes evidence, in addition to that already presented (5), that the isotope was incorporated into the structure of the virus. The nucleic acid fraction had a specific activity about four times that of the phospholipid, practically all the P^{32} of the nucleic acids appearing in the pentose nucleic acid. At the beginning of the work it was hoped that labelled influenza virus might be of assistance in determining whether the nucleic acids of the virus were synthesized after infection of the cell or incorporated into the virus directly from the constituents of the cell. However, the low specific activities of the radioactive virus preparations so far obtained, in addition to the relatively short half life of P^{32}, make analytical

work difficult. A method must be found for greatly increasing the specific activity of the virus before the problem will be amenable to this method of attack.

Acknowledgment

The author is greatly indebted to Dr. Laurella McClelland for providing numerous preparations of purified influenza virus.

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Reprinted from Canadian Journal of Research, E, 28 : 271-280. December, 1950

THE FRACTIONATION OF PHOSPHORUS CONTAINING CONSTITUENTS IN THE ALLANTOIC MEMBRANE OF THE EMBRYONATED EGG[1]

By A. F. Graham

Abstract

A procedure is described which permitted the phosphorus containing constituents in allantoic membranes of embryonated eggs to be separated into fractions as follows: alcohol soluble phosphorus from which the phospholipids were separated, phosphorus soluble in 5% trichloroacetic acid in which inorganic phosphorus was determined, and nucleic acid phosphorus which was further separated into pentose and desoxypentose nucleic acids. This procedure was applied to membranes between 9 and 13 days of age. It was found that the total phosphorus amounted to 9 to 10 mgm. per gm. of dried tissue. Alcohol soluble phosphorus accounted for approximately 28%, acid soluble phosphorus for 37%, and nucleic acid phosphorus for 35% of the total phosphorus. About half the acid soluble phosphorus was inorganic and about 85% of the alcohol soluble phosphorus was associated with phospholipids. These proportions remained essentially constant over the period studied. The ratio of pentose to desoxypentose nucleic acid phosphorus also remained fairly constant over the interval at about 2.2. There was no significant difference in the amounts of P^{32} taken up over a period of 72 hr. by normal allantoic membranes and those infected with influenza virus, when inorganic radioactive phosphorus was placed in the allantoic sacs of 11-day embryonated eggs.

Introduction

Previous work has demonstrated (3) that influenza virus A growing in the allantoic membrane of the embryonated egg in the presence of radioactive inorganic phosphorus incorporated the isotope into its structure. Both the nucleic acid and phospholipid components of the virus were found to contain the isotope (2).

In view of these findings the question arose as to whether nucleic acid and phospholipid already present in the cell were incorporated into the virus or whether these components of the virus were synthesized after infection of the cell. As an aid in answering this question, it was considered that some information should be obtained on the rate of uptake of P^{32} by the constituents of normal cells.

Prior to this study it was necessary to determine the distribution of phosphorus among the various components of allantoic membrane. The present paper describes the techniques employed to separate the phosphorus of allantoic membrane into fractions soluble in alcohol, 5% trichloroacetic acid, and a residue containing nucleic acid and phosphoprotein. Further, the phospholipid present in the alcohol soluble fraction, inorganic phosphorus in the trichloroacetic acid soluble fraction, and the proportion of pentose to desoxypentose nucleic acid were estimated. This procedure, which, in many respects,

[1] *Manuscript received July 24, 1950.*

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is similar to that described by McCarter and Steljes (4) for rat tissue, was applied to membranes collected from fertile eggs 9 to 13 days of age and the results are described.

Some preliminary results on the uptake of P^{32} by allantoic membranes before and after infection with influenza virus are also described.

Methods

Estimation of Total Phosphorus

The method for determining total phosphorus has been described in a previous paper (3).

Estimation of Radioactive Phosphorus

A liquid Geiger-Müller counter, capacity 10 ml., as described by Veall (7) was used in conjunction with a conventional scale of 64 unit. The counter was calibrated against a standard Ra D + E source from the National Bureau of Standards, Washington, as previously described (2). In this paper the activities of radioactive sources are recorded as counts per minute (c.p.m.) 1000 c.p.m. being equivalent to 86 μrd.

Preparation of Allantoic Membranes for Analysis

Embryonated eggs from a pure bred White Leghorn flock, previously candled to ensure that they were alive, were opened at the air sac end, an incision was made in the allantoic membrane and the contents of the egg were poured out. Generally the allantoic membrane was left adhering to the inside of the egg; it was rapidly removed, rinsed twice in ice-cold 0.85% sodium chloride (w/v) immediately frozen and stored at $-20°$ C. in a screw capped vial. Before use the material was immersed in liquid nitrogen in a stainless steel mortar, pulverized, dried *in vacuo* from the frozen state and stored over P_2O_5 in a vacuum desiccator. No attempt was made to choose eggs of a particular weight.

Experimental

Extraction of Trichloroacetic Acid Soluble Phosphorus and Alcohol Soluble Phosphorus from Membrane Tissue

To determine whether extraction of dried membrane with 5% trichloroacetic acid (TCA) would influence later extraction of alcohol soluble phosphorus, an experiment was performed in which one quantity of tissue was extracted with boiling alcohol and then with 5% TCA and a second quantity was extracted with 5% TCA followed by extraction with hot alcohol.

Finely powdered tissue, 0.232 gm., was extracted with 5 ml. of boiling absolute alcohol (freshly purified by refluxing over solid potassium hydroxide and then distilling through a fractionating column) for five minutes in a centrifuge tube fitted with a small reflux condenser. After centrifuging the supernatant solution was removed and made to 25 ml. volume. This procedure was repeated twice more on the residue, and phosphorus estimations were carried out on each extract.

The extracted residue was allowed to dry at 37° C. and homogenized in 5 ml. of ice-cold 5% TCA for two minutes in a homogenizer of the type described by Potter and Elvehjem (5). During this procedure the homogenizer was surrounded with crushed ice. The mixture, with 2 ml. of 5% TCA used to wash out the grinder, was centrifuged and the supernatant made to 25 ml. volume. The residue was extracted twice more with 5% TCA in the same manner and phosphorus analyses were carried out on each extract.

In the second part of the experiment the above extraction sequence was reversed. The tissue, 0.232 gm., was extracted three times with 5 ml. of 5% TCA as above. The residue was resuspended in 5 ml. of absolute alcohol to remove residual TCA, centrifuged, and this was repeated. The two alcohol washes were combined and made to 25 ml. This solution is designated "1st alcohol extract". Two further extractions of the tissue with boiling absolute alcohol for five minutes each were carried out and the three extracts were analyzed for phosphorus.

The results of this experiment are shown in Tables I and II. It is seen that the amount of phosphorus extracted from the tissue by boiling alcohol or 5%

TABLE I

AMOUNTS OF PHOSPHORUS PRESENT IN SUCCESSIVE ALCOHOL EXTRACTS OF ALLANTOIC MEMBRANE BEFORE AND AFTER EXTRACTION WITH TRICHLOROACETIC ACID, 0.232 GM. DRY TISSUE USED IN EACH CASE

Fraction	Alcohol extracts before TCA extraction of tissue, μgm. P	Alcohol extracts after TCA extraction of tissue, μgm. P
1st extract	380	373
2nd extract	53	66
3rd extract	14	2

TABLE II

AMOUNTS OF PHOSPHORUS PRESENT IN SUCCESSIVE TRICHLOROACETIC ACID EXTRACTS OF ALLANTOIC MEMBRANE BEFORE AND AFTER EXTRACTION OF THE TISSUE WITH ALCOHOL, 0.232 GM. DRIED TISSUE USED IN EACH CASE

Fraction	5% TCA extracts before alcohol extraction of tissue, μgm. P	5% TCA extracts after alcohol extraction of tissue, μgm. P
1st extract	525	508
2nd extract	40	35
3rd extract	7	5

TCA was the same regardless of which solvent was employed first. Further, three extractions of the tissue with either solvent were sufficient to remove all the phosphorus compounds soluble in that solvent. This observation was

amply confirmed in other experiments. In some experiments a mixture of alcohol and ether (3/1, v/v) was used instead of absolute alcohol alone with similar results.

During extraction with TCA in the glass homogenizer a considerable amount of glass was ground off and became mixed with the tissue. Control experiments showed that this glass did not interfere in the phosphorus estimation.

Separation of Phospholipids from the Alcohol Soluble Fraction of Allantoic Membrane

An aliquot of the alcohol extract of tissue was evaporated to dryness at 30° C. in a stream of nitrogen. The residue was extracted three times for 10 min. each with warm petroleum ether (b.p. 40° – 60° C.) and the extracts, containing the phospholipids, were combined and analyzed for phosphorus.

To determine whether extraction of the tissue with 5% TCA prior to extraction with alcohol influenced the separation of phospholipid, the above procedure was applied to two alcohol extracts of membrane. The first alcohol extract was obtained after the tissue had been extracted with 5% TCA and the second was prepared directly from dried tissue. In both cases phosphorus analyses were carried out on the petroleum ether soluble fraction (phospholipid) and on the petroleum ether insoluble residue, the results being shown in Table III.

TABLE III

SEPARATION OF PETROLEUM ETHER SOLUBLE PHOSPHORUS FROM ALCOHOL SOLUBLE FRACTION OF DRIED ALLANTOIC MEMBRANES

Fraction	Alcohol extract prepared before TCA extraction of membranes, μgm. P	Alcohol extract prepared after TCA extraction of membranes, μgm. P
Petroleum ether soluble	34.2	20.2
Petroleum ether insoluble	8.0	22.0
Total alcohol soluble P added	45.7	44.7

It would appear that separation of phospholipid was less complete in the case where the membrane had been treated with TCA prior to alcohol extraction. It is noteworthy that this alcohol extract contained three to four times the amount of solid material found in the other alcohol extract. It was concluded that the phospholipids were more difficult to separate from the alcohol soluble material when the tissue had been subjected to previous treatment with TCA.

Estimation of Inorganic Phosphate in Trichloroacetic Acid Extracts of Membrane

In estimating inorganic phosphorus in trichloroacetic acid extracts of tissue the usual colorimetric method (3) was applied to the extracts without prior

acid digestion. To determine whether the sulphuric acid and molybdate present in the reagent might liberate inorganic phosphorus from easily hydrolyzable compounds in the extracts, the following experiment was performed.

A small amount of dried tissue was extracted in the cold with 5% TCA. Aliquots of the extract were added to three tubes containing molybdate and sulphuric acid, in the proportions required by the method, and the volumes made up to about 13 ml. with water. The color was developed in the first tube immediately by addition of stannous chloride solution and in the other tubes when they had stood 15 min. and 30 min. respectively at room temperature. The amounts of phosphorus estimated in the three tubes were identical.

A further experiment was carried out to find whether inorganic phosphorus might be liberated by the action of TCA during preparation of the extracts. A small quantity of tissue was extracted as rapidly as possible in the cold with 5% TCA. After centrifuging in the cold an aliquot was taken immediately from the supernatant solution for estimation of inorganic phosphorus. The remaining extract was kept at 37° C. and estimations of inorganic phosphorus were carried out at intervals over a period of 90 min. There was no change in the amount of inorganic phosphorus estimated over this period.

As a further check, the inorganic phosphorus contents of several TCA extracts of tissue were estimated using the modification of the method of Delory (1) described in a previous paper (2). Inorganic phosphorus was also estimated directly in these extracts as described above. On the average the amount of inorganic phosphate found from the direct estimation was about 10% higher than that found with Delory's method.

Estimation of Pentose Nucleic Acid (PNA) and Desoxypentose Nucleic Acid (DNA) in Allantoic Membrane

The method used was that of Schmidt and Thannhauser (6) and as applied in the present work was as follows. To tissue (0.100–0.250 gm.) which had been extracted with alcohol and 5% TCA, as described in preceding sections, was added 4.0 ml. of 1.0 *N* potassium hydroxide. The mixture was incubated 18–20 hr. at 37° C., during which time the tissue went into solution, and was then centrifuged to remove ground glass resulting from the preliminary TCA extraction in the homogenizer. Aliquots of supernatant were taken for estimation of total nucleic acid phosphorus. To 3 ml. of supernatant was added 3 ml. of 5% TCA and 0.6 ml. of 6 *N* hydrochloric acid. The precipitated DNA and protein was centrifuged off and washed once with 2.5 ml. of 5% TCA. This wash was added to the first supernatant, the total volume was made to 10 ml. and phosphorus analyses were carried out to determine PNAP. About 1 ml. of 0.1 *N* potassium hydroxide was added to the DNA precipitate which was dissolved by warming, the volume was made up to 10 ml., and the DNAP content was estimated by phosphorus analysis.

To determine the efficiency of the method in separating DNA and PNA, two samples of lipid free, TCA extracted tissue of equal weight were taken.

To one of these were added known amounts of calf thymus nucleic acid an yeast nucleic acid. Both samples were submitted to the separation procedur described above. The results are shown in Table IV.

TABLE IV

SEPARATION OF YEAST NUCLEIC ACID AND THYMUS NUCLEIC ACID, ADDED TO LIPID FRE TRICHLOROACETIC ACID EXTRACTED ALLANTOIC MEMBRANE TISSUE, BY THE METHOD OF SCHMIDT AND THANNHAUSER

	Total phosphorus, μgm.	PNA phosphorus, μgm.	DNA phosphorus, μgm.
Membrane (0.118 gm.) with added PNA and DNA	468	297	168
Membrane (0.118 gm.) alone	324	230	92
Recovered	144 (94%)	67 (93%)	76 (94%)
Added	153	72	81

It is seen that the recovery of added PNAP and DNAP was 93 to 94% of that added.

On several occasions DNAP and PNAP, after separation by the Schmid and Thannhauser method, were estimated by the diphenylamine and orcino color reactions as previously described (2). The diphenylamine reaction wa applied after the DNA had been extracted from the protein with 5% TC for 45 min. at 95° C. This extraction removed all but 3% of the phosphoru of the DNA fraction.

The results obtained with these color tests varied a good deal, the orcino reaction indicating 15 to 35% more PNAP than direct phosphorus estimatio and the DNAP being 12 to 25% higher by the diphenylamine reaction tha by phosphorus analysis.

Distribution of Phosphorus Constituents in Allantoic Membranes

As a result of the above experiments the following procedure was adopte to fractionate the phosphorus containing constituents of allantoic membrane

The dry weighed material, 100 to 350 mgm., was extracted with boilin absolute ethanol, or ethanol–ether (3/1, v/v), phosphorus analyses bein carried out on the extract. An aliquot of the alcohol extract was evaporate to dryness and thoroughly extracted with petroleum ether, to remove phosph lipids, and this solution was analyzed for phosphorus. The alcohol insolubl material was homogenized with 5% TCA, total and inorganic phosphoru being estimated in the extract. The Schmidt and Thannhauser technique wa applied to the residue to separate the nucleic acids. PNAP as estimated her includes the phosphorus from any phosphoprotein which may have bee present in the membrane, no attempt being made to estimate the phosph protein separately.

This analytical procedure was applied to membranes collected at 24 hourly intervals from embryonated eggs 9 to 13 days of age. Seven or eight membranes were collected at each time, pooled, and analyzed together. The results are shown in Figs. 1, 2, and 3. In Fig. 1 the phosphorus contents of the various

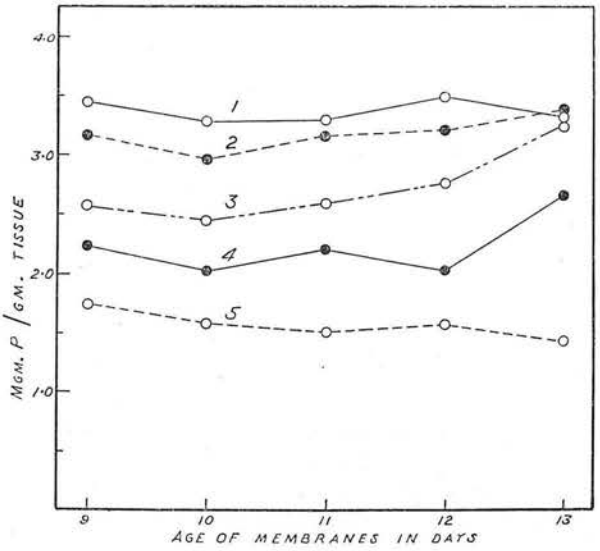

FIG. 1. *Phosphorus contents in mgm. per gm. of dry tissue of the various fractions of allantoic membranes between 9 and 13 days of age. Curve 1, 5% trichloroacetic acid soluble phosphorus. Curve 2, total nucleic acid soluble phosphorus. Curve 3, alcohol soluble phosphorus. Curve 4, petroleum ether soluble phosphorus (phospholipid). Curve 5, inorganic phosphorus, present in 5% trichloroacetic acid soluble fraction.*

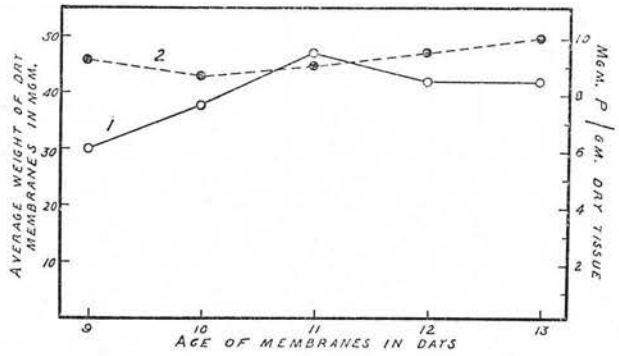

FIG. 2. *Relationship of average dry weight of allantoic membranes, Curve 1, and total phosphorus content, Curve 2, to age of membranes.*

fractions calculated in terms of mgm. P per gm. dry tissue are plotted against the age of the membrane in days. In Fig. 2 are plotted the average weights of the membranes in mgm. and the total phosphorus content of the membranes as mgm. per gm. dry tissue. The figures for the total phosphorus contents of the membranes were obtained by addition of the results from the individual

fractions shown in Fig. 1. Fig. 3 shows the ratio of PNAP (including phosphoprotein phosphorus) to DNAP, obtained in three separate experiments, plotted against age of membrane.

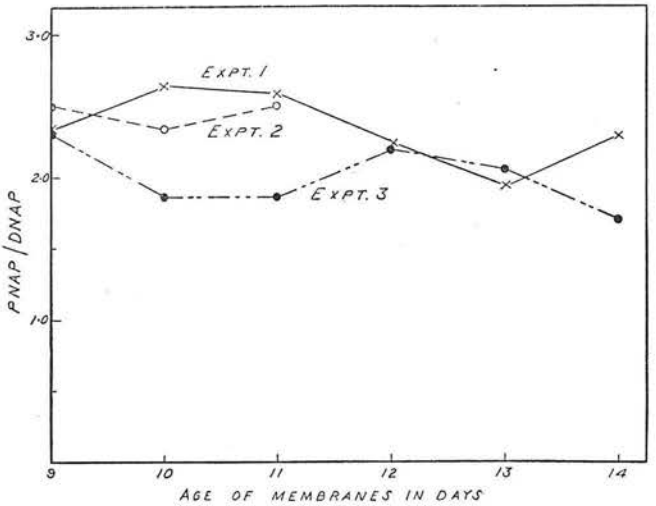

FIG. 3. *The ratio of pentose nucleic acid phosphorus, PNAP, to desoxypentose nucleic acid phosphorus, DNAP, in allantoic membranes between 9 and 14 days of age.*

The results shown in Figs. 1 and 2 for a single experiment were similar to those obtained in a second complete experiment carried out on membranes 9 to 14 days of age.

Uptake of Radioactive Phosphorus by Normal Allantoic Membranes and Membranes Infected with Influenza Virus A

The general procedure for determining the uptake of P^{32} by allantoic membranes was as follows. P^{32}, as inorganic phosphate in 0.85% sodium chloride solution, was injected from a calibrated syringe into the allantoic fluid of 25 to 30 11-day embryonated eggs, 0.2 ml. containing 3000 to 4000 c.p.m. into each egg. The eggs were then incubated at 36° C. and at intervals the membranes from a group of five or six eggs were collected. After being washed thoroughly in two changes of ice-cold saline, the membranes were digested in a mixture of 5 ml. of concentrated sulphuric acid and 2 ml. of 72% perchloric acid and the volume was made up to 50 ml. Radioactivity estimations were carried out on these solutions.

In one experiment with 11-day-old embryonated eggs the uptake of P^{32} by normal membranes was determined over a period of 115 hr. and the results are shown in Fig. 4 as Curve 3. A second experiment was carried out in which two groups of 25 11-day eggs were chosen at random from a large number. Influenza virus A (PR8 strain) was injected in 0.2 ml. amount (10000 ID_{50}) by the allantoic route into each egg of the first group. P^{32} was then injected

into all the eggs which were incubated at 36° C. The P^{32} contents of the membranes of each group were determined at intervals as described above and the results are plotted in Fig. 4, as Curves 1 and 2.

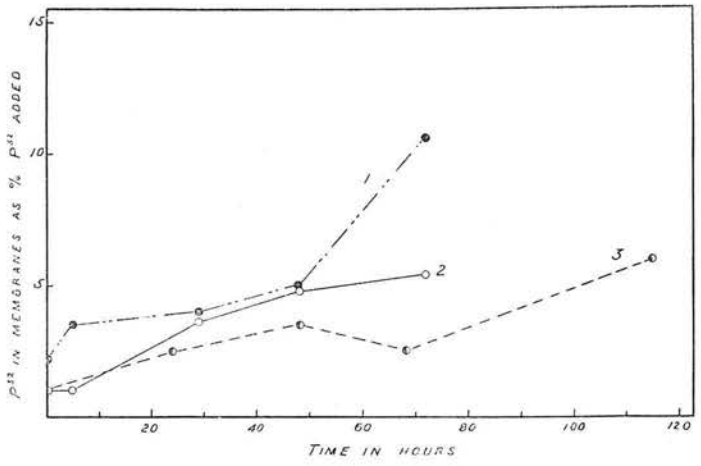

FIG. 4. *Uptake of P^{32} by allantoic membranes from inorganic radioactive phosphorus placed in the allantoic sacs of 11-day embryonated eggs. Curve 1 represents uptake of P^{32} for eggs infected with influenza virus and Curve 2 for normal embryonated eggs, the experiments being carried out simultaneously. Curve 3 represents uptake of P^{32} by normal membranes in a separate experiment.*

Discussion

The procedure described permitted the phosphorus constituents of allantoic membrane to be separated into several fractions, acid soluble phosphorus in which the inorganic phosphorus was determined, alcohol soluble phosphorus from which the phospholipids were separated, and nucleic acid phophorus which was further divided into PNAP and DNAP.

When this procedure was applied to membranes between 9 and 13 days of age the amounts of acid soluble, inorganic, alcohol soluble, phospholipid, nucleic acid, and total phosphorus per gram of tissue remained essentially constant over this interval. Acid soluble P accounted for about 37%, alcohol soluble P for about 28%, and nucleic acid P for about 35% of the total phosphorus of the membrane. In a second such experiment the figures were 46%, 21%, and 33% respectively. The total phosphorus in the membranes amounted to 9 to 10 mgm. per gm. of dried tissue. About half the TCA soluble P was inorganic and about 84% of the alcohol soluble P was contained in phospholipids. Since the membranes on the average increased about 80% in weight between 9 and 11 days the actual amounts of the various phosphorus constituents increased by roughly the same proportion.

Although there was considerable variation in the PNAP/DNAP ratio this ratio may be greatly influenced by small errors in analysis and it is considered that it remains essentially constant at about 2.2 over the 9 to 14 day period.

It is doubtful whether there was any significant difference between the amounts of P^{32} taken up by normal and virus infected 11-day membranes. However, further work may show that there is a significant difference in the rates with which the various components of the membrane are labelled in the two cases. It would be of interest to determine whether the rate of uptake is faster by nine-day membranes than by those 11 days of age since the membrane weight and phosphorus content increase roughly twofold between 9 and 11 days. It seems possible that the P^{32} in the allantoic fluid is not readily utilized by the membrane cells. If this is so, it would be worthwhile to determine the main source of the phosphorus utilized by these cells. A method might then be devised for increasing the specific radioactivity of influenza virus grown in the membrane.

Acknowledgment

The author is indebted to Mrs. Anna Plucis for technical assistance.

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The Toxicity of P^{32} for Normal and Influenza Virus Infected Embryos[1]

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In view of the increasing interest being taken in the labelling of viruses with radioactive isotopes it is of considerable importance to have some knowledge of the toxicity of these isotopes for chicken embryos. During an investigation carried out during 1948 and 1949 by Graham and McClelland (Can.J.Research, E, 28, 121, 1950) on the uptake of P^{32} by influenza virus, some observations were made on the toxic effect of P^{32} in embryonated eggs. For 11 day old embryos, inoculated by the allantoic route with the PR8 strain of influenza virus A, the average number of deaths was found to be 3 to 4 per cent over a period of 48 hours. When P^{32} as inorganic phosphate, obtained from Chalk River, was injected by the same route shortly after virus the embryo death rate amounted to as much as 30 per cent in 48 hours with 0.15 microcuries per egg. It was suggested that the deaths were caused by radiation damage although the possibility that some material toxic to the embryos might be present in the P^{32} was also discussed. In work carried out during the same period Taylor and Saenz (J. Immunol., 63, 319, 331, 1949) injected up to 0.3 microcuries per egg, and Ward (Am. J. Hyg., 52, 107, 1950) used up to 8 microcuries

[1]Aided by a grant from the W. B. Boyd Memorial Fund

per embryo. Both groups of workers employed isotopes obtained from Oak Ridge.

Several months ago we were informed by Dr. Harvey Blank that his group at the Children's Hospital, Philadelphia, had carried out a study on the toxicity of P^{32} for embryonated eggs. Up to one millicurie per egg was used without deleterious effect, similar results being obtained with material from Oak Ridge and Chalk River. In view of the very large discrepancy between our results and the findings of the Philadelphia workers we were led to reinvestigate this problem. The present paper describes an experiment designed to test the toxicity of P^{32}, currently produced at Chalk River, in both normal and PR8 virus infected eggs up to the level of 100 microcuries per egg.

The techniques were similar to those employed by Graham and McClelland (1950). P^{32}, obtained from Chalk River, as phosphoric acid, carrier free, was adjusted to pH 7 and sterilized at 100°C for 30 minutes. This was diluted with 0.85 per cent sodium chloride buffered to pH 7 to give four solutions containing 107, 10.7, 1.07 and 0.1 microcuries P^{32} per 0.2 ml respectively. Two tenths ml of each P^{32} solution was injected into the allantoic sacs of a group of 40 11-day old chick embryos. Twenty eggs from each group had just previously been inoculated with 0.2 ml PR8 virus by the allantoic route, the remaining twenty eggs from each group had received 0.2 ml buffered saline. Of a control group of 40 eggs, 20 were inoculated with 0.2 ml saline and the remainder received 0.2 ml PR8 virus per egg. Each egg of

the control group was then injected with 0.2 ml. saline. All eggs were candled twice daily and the deaths recorded (table 1). The allantoic fluids of all dead embryos were tested for sterility.

Over the 48 hour period of observation, the death rate of normal or PR8 infected 11 day embryos injected with up to 107 microcuries P^{32} per embryo was not significantly increased over that of the controls. Although 5 out of the total of 19 deaths could have been attributed to bacterial contamination, the number of deaths varied considerably from one group to another, including the controls, and cannot be related to any one responsible factor. It is concluded, therefore, that the high death rate observed previously with very much smaller amounts of P^{32} did not result from radiation damage but perhaps from the presence of some unknown toxic factor in the isotope.

It was found by Graham and McClelland that with less than 0.21 microcuries P^{32} per egg the specific activity of labelled virus rose in a linear manner with the amount of administered isotope. If this linear relationship extends to the 100 microcurie level it should be feasible to obtain purified labelled influenza virus with specific activity at least 100 to 1000 fold higher than that previously achieved.

TABLE 1

Death Rate of Normal and Influenza Virus Infected Embryos after Administration of Varying Amounts of P^{32}

Group	Number of Deaths, Hours after Infection					Total Deaths in 65 Hours
	17	24	41	48	65	
20 eggs, 107 microcuries P^{32} + PR8	1	-	2	-	-	3/20
20 eggs + 107 microcuries P^{32}	-	-	2	-	2	4/20
20 eggs + 10.7 microcuries P^{32}+PR8	1	-	-	-	-	1/20
20 eggs + 10.7 microcuries P^{32}	2	-	2	1	-	6/20
20 eggs + 1.1 microcuries P^{32}+PR8	1	-	-	-	1	2/20
20 eggs + 1.1 microcuries P^{32}	2	-	-	-	-	2/20
20 eggs + 0.1 microcuries P^{32}+PR8	-	-	-	-	-	0/20
20 eggs + 0.1 microcuries P^{32}	-	-	-	1	-	1/20
20 eggs + PR8 + 0.2 ml saline	2	-	1	1	-	4/20
20 eggs + two injections of 0.2 ml saline	-	-	-	-	-	0/20

Reprinted from ARCHIVES OF BIOCHEMISTRY
Vol. 28, No. 1, August, 1950
Printed in U. S. A.

Breakdown of Infecting Coliphage by the Host Cell [1]

The present letter is a report of work carried out with T_2r+ bacteriophage, labeled with P^{32}, active on *Escherichia coli* B, to determine the fate of the infecting particle. The virus was labeled and purified in a manner similar to that described by Putnam and Kozloff (1) for T_6 bacteriophage, the P^{32} being contained almost entirely in the nucleic acid of the virus.

Cultures of *E. coli* grown in Difco Bacto-Tryptose broth to 2×10^8 cells/ml. were infected with the labeled bacteriophage and aerated at 37° C. At intervals measured amounts of the culture were withdrawn and concentrated trichloroacetic acid (TCA) was added to make a final concentration of 5%. The mixture was centrifuged and the amount of P^{32} in the supernatant was used as a criterion of the extent of breakdown of nucleic acid in the infecting virus particle. The amount of P^{32} present in the TCA extract was calculated as a percentage of the P^{32} added in the infecting bacteriophage. Figure 1 illustrates average curves relating this quantity to time after addition of radioactive bacteriophage to the cells for different experimental conditions.

Curve I shown the release of P^{32} when cells were singly infected with radioactive bacteriophage, 1 virus particle to 5 cells, and curve II when 2 to 10 virus particles/cell were added to the culture. When cells were infected with purified but not radioactive T_2r+ bacteriophage, and labeled bacteriophage was added at some later time, the breakdown of labeled bacteriophage was as shown in curve III. Curve III was reproduced whether the initial infection was 2 to 10 virus particles/cell or the labeled bacteriophage was added at ratios of 0.2 to 60 particles/cell between 2–120 min. following initial infection.

The following interpretation is proposed tentatively. Infection of the cells stimulates some mechanism, at present unknown, so that other virus particles becoming attached to the cell after a short interval are each broken down to the extent of about 52% as shown in curve III. The toe of curve I represents the conditions when the cell is singly infected, namely, about 4% breakdown of the infecting particle. Multiple infection as shown by curve II represents an intermediate state; the secondary rise of curve I might then be explained as a multiple infection of the type of curve II resulting from the release of first generation of virus and adsorption on uninfected cells.

When cells were multiply infected with radioactive bacteriophage about 20% of the P^{32} appeared in the progeny. However, when the labeled virus was added 15

[1] This investigation was aided by a grant from the National Cancer Institute of Canada.

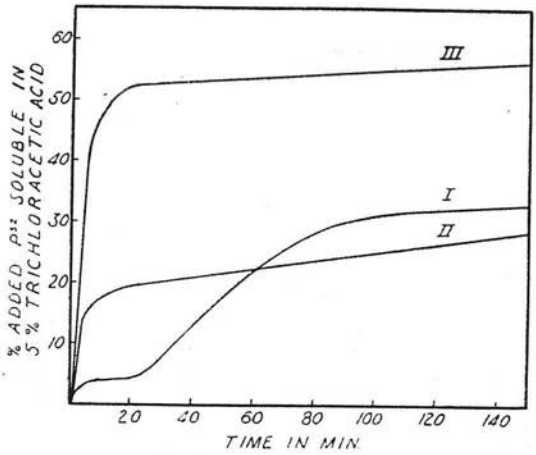

FIG. 1. Breakdown of infecting coliphage by the host cell.

minutes after multiple infection with nonradioactive bacteriophage the progeny contained only 1% of the added radioactivity. Apparently, virus adsorbed after infection is initiated contributes little to the formation of progeny.

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Received June 13, 1950

Reprinted from Canadian Journal of Research, E, 28 : 281-288. December, 1950

STUDIES ON THE RELATIONSHIP BETWEEN VIRUS AND HOST CELL: THE PREPARATION OF T2r^+ BACTERIOPHAGE LABELLED WITH RADIOACTIVE PHOSPHORUS[1]

By S. M. Lesley, R. C. French, and A. F. Graham

Abstract

T2r^+ bacteriophage grown in its host, *Escherichia coli* B, in broth medium in the presence of radioactive inorganic phosphorus was labelled with the isotope. Purified suspensions of this virus had specific activities up to 50,000 c.p.m. per μgm. P. There was little or no exchange of P^{32} between virus and inorganic phosphate. Chemical analysis showed that at least 98% of the virus phosphorus was contained in nucleic acid; of the nucleic acid phosphorus 95.5% was associated with desoxypentose nucleic acid and 4.5% with pentose nucleic acid. More than 99% of the radioactivity of the labelled bacteriophage was contained in the nucleic acid fraction. Preparations of bacteriophage were obtained with sufficiently high specific activity to enable metabolism experiments to be carried out on the growth of the labelled virus in the host cell.

Introduction

In a previous paper (6) a method was described which enabled influenza virus growing in the embryonated egg to be labelled with radioactive phosphorus. As was pointed out the main purpose of that work was to obtain highly radioactive specimens of influenza virus to enable the metabolism of the isotope to be studied when cells were infected with the labelled virus. While little difficulty was experienced in labelling influenza virus with relatively small amounts of P^{32}, the specific activity was not sufficiently high to enable the metabolism experiments to be carried out.

It was considered, however, that such a study might be more feasible with a different virus – host cell system, namely T2 bacteriophage active on *Escherichia coli*, strain B. It has already been demonstrated by Cohen (1) that the T2 and T4 bacteriophage of *E. coli*, and by Kozloff and Putnam (10) that the T6 bacteriophage were labelled with P^{32} when inorganic radioactive phosphorus was added to the medium supporting virus growth in the infected cells.

The present paper describes the methods used to obtain purified highly radioactive preparations of T2r^+ bacteriophage labelled with P^{32}. Experiments are described which support the conclusion that the label is contained almost entirely in the nucleic acid fraction of the virus.

Utilizing these methods preparations of bacteriophage were obtained sufficiently high in specific radioactivity to enable metabolism experiments to be carried out with the virus on its host, *E. coli*, strain B.

[1] *Manuscript received July 17, 1950.*

Contribution from Connaught Medical Research Laboratories, University of Toronto. Aided by a grant from the National Cancer Institute of Canada.

Methods and Materials

Determination of Total Phosphorus

The method of estimating total phosphorus has been previously described in detail (6).

Determination of Radioactive Phosphorus

The radioactivity of P^{32} solutions was estimated with a liquid Geiger-Müller counter, capacity 10 ml., of the type described by Veall (13). The counter was standardized against a RaD+E source from the National Bureau of Standards, Washington, as previously described (5). The results of radioactivity measurements, given in this paper in terms of counts per minute (c.p.m.), may be converted to disintegrations per second using the factor 1000 c.p.m. are equivalent to 86 μrd.

Specific activity is defined as c.p.m. per μgm. P unless otherwise indicated.

Preparation of Media

The liquid medium used throughout this work was a nutrient solution prepared by dissolving 20 gm. Difco Bacto–Tryptose, 5 gm. sodium chloride, and 1 gm. glucose per liter in distilled water. After adjusting the pH to about 7.2, the solution was autoclaved. Solid medium was prepared by adding agar to the broth medium before autoclaving.

Type of Bacteriophage and Bacteria

The bacteriophage used throughout was the T2r^+ strain, active on *E. coli* B, received from Dr. Fred Heagy, University of Western Ontario. Using the method of Delbrück and Luria (3) this bacteriophage was found to have a latent period of 23 min. and a burst size of 115 in Bacto–Tryptose broth.

Determination of Bacterial Concentration in Liquid Cultures

The concentration of cells in broth culture was estimated on a Coleman Spectrophotometer at 630 mμ, the per cent transmittance being read against broth as a blank. The instrument was standardized against cell suspensions in which the number of cells was determined by colony count and also by direct count in a Petroff–Hausser counting chamber.

Determination of Virus Concentration

The number of infective particles in a bacteriophage suspension was determined by the method of Hershey *et al.* (7). Since slight modifications were made to the original method, the procedure is described below.

Serial 10- or 100-fold dilutions of the virus solution were made in 0.85% (w/v) sodium chloride (saline) until the number of infective centers in the final dilution was between 60 and 1200. To 1 ml. of this dilution was added 3 ml. of broth suspension of *E. coli* containing about 2×10^8 cells per ml., freshly prepared from a 15–20 hr. agar slope. One ml. aliquots of this mixture were then added to each of three tubes containing 1 ml. of melted nutrient agar (0.7%). The contents of each tube were mixed and poured over the surface

of a nutrient agar (1%) plate. When the agar had solidified the plates were incubated for 18 hr. at 37° C. and the plaques were counted. From the results the number of plaque forming particles per ml. of the original suspension was calculated and in this paper is expressed as phage per ml. The plating efficiency determined by the method of Ellis and Delbrück (4) was about 0.65.

When titrating saline suspensions of bacteriophage, prepared as described in a later section, a 100-fold dilution of the suspension was made in broth and allowed to stand at least one hour before further dilutions were made in saline. It was found that higher titers were obtained by this procedure than when the initial dilution was made in saline.

Experimental

Growth and Purification of Bacteriophage

Cultures of *E. coli* B were prepared by inoculation of 150 ml. of broth from an 18 hr. slope and grown to 2×10^8 cells per ml. with rapid aeration at 37° C. The generation time of *E. coli* under these conditions was about 21 min. After centrifuging, the cells were resuspended in 150 ml. of fresh broth at 37° C. and immediately inoculated with T2r^+ bacteriophage from a stock broth culture usually in the ratio of three to five virus particles per cell. In some cases, for example in preparing phage labelled with P^{32} as described later, the centrifuging and resuspension of the cells were omitted and the virus inoculum was added to the culture as soon as it has reached the required concentration of cells.

The culture was rapidly aerated at 37° C. until lysis occurred when the titer of phage reached 4×10^{10} to 10^{11} phage per ml. Bacterial debris was then removed by centrifuging at 4300 g. for 30 min. in a Sorvall angle centrifuge at 5° C. About 25% of the virus was lost in this step, but further purification depended upon efficient removal of the debris. The virus was then sedimented by centrifuging the supernatant solution at 22,000 g. for one hour in the angle centrifuge. The supernatant was aspirated off until the level of liquid was just above the virus pellet which adhered to the side of the tube. When the tube was left for some time a bluish material was observed to separate slowly from the pellet and fall to the bottom of the tube. By this means a separation was obtained between virus and the nonviral material which remained adhering to the tube as an opaque tightly packed pellet. When the separation was complete, after 20–40 min., as much as possible of the remaining liquid and the pellet were carefully drawn off through a fine tipped pipette leaving the virus in the bottom of the tube as a small opalescent pool. A few ml. of 0.85% saline were then added and the virus was dispersed by giving the tube a quick swirl. The volume was made up with saline to about one-quarter the original volume, the virus was sedimented at 22,000 g. and separated from the pellet as before. The nonviral pellet was very small at this stage and was absent when the procedure was repeated a third time. The virus was finally resuspended in 10 ml. of saline, the recovery being 25 to 40% of that

present in the original lysate. With this procedure the customary intermediate low speed centrifugations to remove aggregated material after resuspension of the virus were unnecessary.

Properties of the Purified Virus

Preparations of purified virus in 0.85% saline containing 10^{11} to 4×10^{12} phage per ml. gave single boundaries in the ultracentrifuge (Fig. 1) with an average sedimentation constant of 1040 S in agreement with the results of Sharp *et al.* (11).

Chromium shadowed preparations in the electron microscope showed the usual tadpole-shaped particles (8), the dimensions of the head being approximately 90 by 125 mμ, the length of the tail about 110 mμ.*

Phosphorus analyses carried out on six preparations of purified phage gave 2.76, 2.67, 4.15, 2.94, 2.15, 3.46×10^{-11} μgm. P per phage or an average of 3.02×10^{-11} μgm. P per phage.

Saline suspensions of purified bacteriophage, kept at 5° C., did not decrease in titer over a period of eight weeks.

Preparation of Bacteriophage Labelled with Radioactive Phosphorus

In order to obtain virus of high specific activity the phosphorus content of the broth medium was decreased as follows. Twenty gm. of Bacto–Tryptose was dissolved in about 200 ml. of distilled water, the solution was adjusted to pH 8.4 and 2 ml. saturated calcium chloride solution was added. The precipitate of calcium phosphate was removed by centrifugation, sodium chloride and glucose were added in the required proportions to the supernatant solution, the volume was made to 1000 ml., and the solution autoclaved. By this means the total phosphorus content of the final medium was reduced from 140 to about 30 μgm. P per ml.

To 150 ml. of this medium was added up to 1.5 mc. of P^{32} as inorganic phosphate, carrier free. The medium was inoculated with *E. coli* from an 18 hr. slope and grown to 2×10^8 cells per ml. at 37° C. with aeration. Bacteriophage from a stock culture was then added to give a final concentration of 6 to 8×10^8 phage per ml., and the suspension was aerated until lysis occurred.

The bacteriophage, which reached a final titer of 4×10^{10} to 10^{11} phage per ml. in the lysate, was purified in the manner previously described. The virus was sedimented repeatedly, usually four to six times, until the specific activity of the supernatant solution, c.p.m. per phage, reached that of the resuspended virus. After each sedimentation the phage was resuspended in about 40 ml. of saline the final suspension being made in 8 to 12 ml. of saline. By this means suspensions of purified virus were obtained having titers of 1 to 2×10^{11} phage per ml., and specific activities varying from 500 to 50,000 c.p.m. per μgm. P depending on the amount of P^{32} added to the medium.

* *We are indebted to Dr. G. D. Scott of the Physics Department for the electron micrographs.*

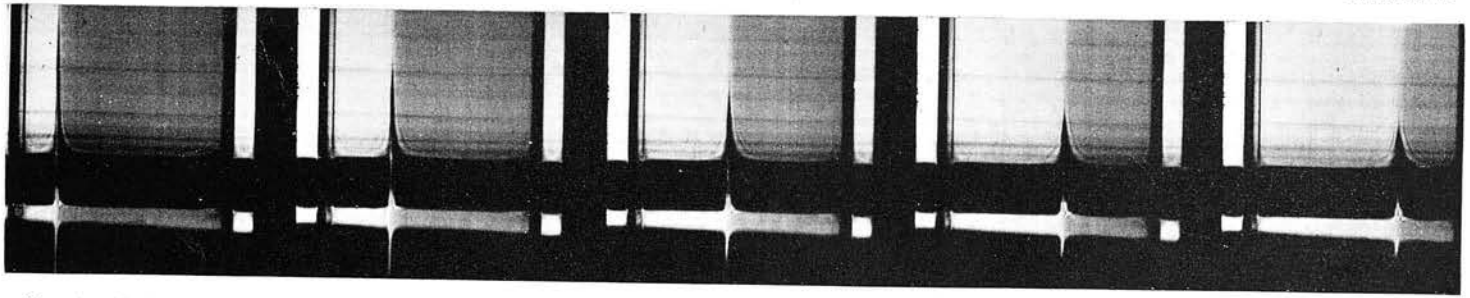

FIG. 1. *Sedimentation diagrams of purified* T2r^+ *bacteriophage in 0.85% sodium chloride at pH 6.05. Concentration was 1.76 mgm. phage per ml. Direction of sedimentation is from left to right. Photographs were taken at two-minute intervals with a mean centrifugal field of 11,100 g. using a Spinco Ultracentrifuge equipped with a Philpot-Svensson type of optical system.*

Since these radioactive preparations were required for other experiments phosphorus analyses were carried out on only a small number. However, there appeared to be no difference in the μgm. P per phage between normal and radioactive preparations. Further, repeated phage titrations carried out at intervals over a period of several weeks with several highly radioactive phage preparations showed no inactivation of virus by the radiation.

Control Experiments to Determine Whether Bacteriophage Takes up P^{32} by Exchange with Inorganic Phosphate

To determine whether P^{32} was taken up by bacteriophage by exchange or adsorption on its surface the following experiment was carried out.

To two tubes, each containing 150 ml. of broth culture of *E. coli*, in the exponential phase of growth, at a concentration of 5×10^7 cells per ml., was added sufficient T2r^+ bacteriophage to give a ratio of approximately 1 virus particle per 40 cells. P^{32} was added immediately to tube A to give a final concentration of 75,000 c.p.m. per ml. Both cultures were then aerated at 37° C. until lysis occurred. An identical amount of P^{32} was then added to tube B as had been added to tube A and both cultures were allowed to stand overnight at 5° C. The concentration of virus in both cultures at lysis was about 7.5×10^{10} phage per ml. The phage was then purified as previously described and total phosphorus and radioactivity estimations were carried out on the final virus suspensions. Purified phage from tube A had a specific activity of 481 c.p.m. per μgm. P, while that from tube B had 1.6 c.p.m. per μgm. P. It was thus apparent that by far the greater proportion of P^{32} contained in radioactive phage was incorporated during growth of the virus in the host cells.

In a further experiment 1.0 ml. of P^{32} (carrier free) solution in saline was added to 1.0 ml. of purified phage to give a final P^{32} concentration of 326,000 c.p.m. per ml. and a specific activity of 28,200 c.p.m. per μgm. P. After standing for 18 hr. at 5° C. the virus was alternately sedimented at 22,000 g. and resuspended in 40 ml. of saline. After the fifth sedimentation the specific activity was found to be 209 c.p.m. per μgm. P, and after the sixth 82 c.p.m. per μgm. P. The final specific activity of the virus was thus 0.3% of the specific activity contained in the initial suspension, indicating little or no exchange or adsorption of radioactive inorganic phosphorus by the virus.

Further Tests to Determine Whether P^{32} was Incorporated into the Structure of Radioactive Bacteriophage

It was thought that if a significant proportion of the P^{32} contained in radioactive bacteriophage were adsorbed on the surface it might be released when the phage was adsorbed on the host cells. To investigate this point purified radioactive phage in broth was added to a broth suspension of *E. coli* to give a ratio of one virus particle to two cells with the final concentration of cells being about 10^{10} per ml. The mixture was kept at 37° C. for seven minutes then chilled to 5° C. and centrifuged to remove the cells and adsorbed bacteriophage. It was found that the supernatant solution thereby obtained contained

14% of the added phage and 16% of the added P^{32}. For all radioactive phage preparations so far tested by this method similar results have been obtained. The isotope would appear to be firmly fixed to the virus particle.

Since trichloroacetic acid is widely used as a solvent to extract inorganic and low molecular weight phosphorus compounds from cells it was of interest to determine whether purified radioactive phage contained phosphorus compounds soluble in trichloroacetic acid. Accordingly, a quantity of purified radioactive phage was added to 20 ml. of broth and allowed to stand an hour or so to ensure that the virus was dispersed. The solution was chilled in an ice bath and 0.2 ml. of dialyzed 1% egg albumin solution was added to act as a protein carrier, followed by 2.9 ml. of 40% trichloroacetic acid. After standing for 15 min. in the cold the precipitate was removed by centrifuging and the P^{32} content of the supernatant determined.

This procedure was carried out on each of nine preparations of purified radioactive phage. The amount of radioactivity remaining in the trichloroacetic acid supernatant was generally less than 0.5% of that added originally with the bacteriophage. In the case of one preparation 0.9% of the added radioactivity was soluble in the trichloroacetic acid. In this particular case a suspension of the purified phage in broth was dialyzed for several days against several changes of 0.1 *M* phosphate buffer pH 7.0. The dialyzate was found to contain 0.9% of the radioactivity present in the bacteriophage.

It is evident from these results that the preparations of purified bacteriophage contained little P^{32} soluble in trichloroacetic acid suggesting that the isotope was incorporated in the structure of the virus.

Chemical Analysis of Radioactive Bacteriophage

In order to obtain additional evidence that the bacteriophage was labelled, a chemical analysis was carried out on a purified radioactive specimen as follows: A preparation of purified radioactive bacteriophage suspended in saline was diluted with about eight times its quantity of purified but not radioactive phage. This gave a suspension containing sufficient virus (about 4.5 mgm.) for analysis and containing an amount of activity convenient for measurement. The preparation contained 2.7×10^{-11} μgm. P per phage and a specific activity of 657 c.p.m. per μgm. P. To the suspension was added 0.25 ml. of 1% egg crystalline albumin to act as a protein carrier. An analytical control, which was treated throughout in the same manner as the virus, contained 0.25 ml. of the albumin solution in saline.

Sufficient 40% trichloroacetic acid was added to give a final concentration of 5% and the precipitate was removed by centrifugation. The residue was washed twice with cold absolute alcohol and then extracted twice with boiling alcohol–ether (3/1, v/v) for 15 min. The residue was then extracted three times at 95° C. with 5% trichloroacetic acid for 15 min. to remove the nucleic

acid. P^{31} and P^{32} estimations were carried out on the 5% trichloroacetic acid, alcohol–ether, and nucleic acid fractions and the nucleic acid free residue; the results are shown in Table I.

TABLE I

CHEMICAL ANALYSIS OF RADIOACTIVE BACTERIOPHAGE

Fractions	Total P^{31}, μgm.	Total P^{32}, c.p.m.	Specific activity, c.p.m. per μgm. P
Whole virus	189	124000	657
5% Trichloroacetic acid soluble	4.2	1175	280
Alcohol-ether soluble	0	50	—
Nucleic acid	210	137000	653
Nucleic acid free residue	0	346	—

It is observed that the phosphorus of the virus is contained almost entirely in the nucleic acid fraction in accordance with the observations of Taylor (12). Further, practically all the radioactivity is associated with the nucleic acid.

Since Taylor (12) has reported the presence of 6.6% pentose nucleic acid and 40.3% desoxypentose nucleic acid in this virus, the orcinol reaction for pentose and the diphenylamine reaction for desoxypentose were applied to the nucleic acid fraction of the virus in the manner described in a previous paper (5). The results of these tests showed the presence of 9.8 μgm. of pentose nucleic acid phosphorus and 204 μgm. of desoxypentose nucleic acid phosphorus, compared to 210 μgm. P in the fraction (Table I) estimated by phosphorus analysis.

Discussion

From sedimentation and electron microscope studies it is concluded that the preparations of purified T2r^+ bacteriophage obtained in this work consisted almost entirely of the virus particles. This conclusion is supported by the finding that one plaque forming unit in the preparations contained about 3.0×10^{-11} μgm. P, a result not inconsistent with the figure of 4.7×10^{-11} μgm. P per phage calculated from the results of Hook *et al.* (8) for this virus and 3.9×10^{-11} μgm. P per phage found by Kozloff and Putnam (9) for the closely related T6r^+ bacteriophage of *E. coli*.

At least 98% of the phosphorus of the virus was contained in the nucleic acid fraction, the remaining 2% being soluble in 5% trichloroacetic acid. About 95.5% of the nucleic acid phosphorus was found in desoxypentose nucleic acid and about 4.5% in pentose nucleic acid. While these results confirm the observation of Taylor (12) that both nucleic acids are present in purified preparations of the virus yet recent work by Cohen (2) indicates that the PNAP found may be present as an impurity. The amount of pentose nucleic acid phosphorus determined by Taylor for this virus by a different method, namely 12.7% of the total nucleic acid phosphorus, was somewhat

higher than obtained in the present work. Kozloff and Putnam (9) have found 2.4 to 3.4% of the total phosphorus in synthetic medium T6r^+ bacteriophage to be present in the nucleic acid.

Sufficient P^{32} was introduced into the virus during its growth to enable metabolism experiments to be carried out with the labelled phage growing on the host cells. That the isotope was, in fact, incorporated into the structure of the virus was indicated by control experiments which showed little or no exchange of P^{32} between inorganic phosphate and virus. Also chemical analysis demonstrated that at least 99% of the P^{32} in the labelled virus was associated with the nucleic acid fraction. Within experimental error the specific radioactivity of the nucleic acid fraction was the same as that of the whole virus.

A small amount of the radioactivity of the virus was soluble in 5% trichloroacetic acid. The proportion varied from one preparation of virus to another up to about 1% but was usually less than 0.5%. It is considered, however, that this was radioactive impurity carried through the purification procedure rather than an integral part of the virus.

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STUDIES ON THE RELATIONSHIP BETWEEN VIRUS AND HOST CELL

II. THE BREAKDOWN OF T2r^+ BACTERIOPHAGE UPON INFECTION OF ITS HOST *ESCHERICHIA COLI*

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Reprinted from Canadian Journal of Medical Sciences, 29 : 128-143. June, 1951

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Abstract

Infection of *E. coli* with P^{32} labelled T2r^+ bacteriophage resulted in the conversion of a part of the isotope to a form soluble in 5% trichloroacetic acid. This acid soluble phosphorus was used as a measure of the breakdown of the virus. When the phage to cell ratio was less than unity a two step breakdown curve was obtained corresponding to the two cycles of infection in this type of experiment. The heights of the steps represented 5 and 32% breakdown, the second rise commencing after about 25 min. Multiple infection of cells yielded a curve which rose without inflection to about 23% breakdown at 20 min. after which there was little further increase. When the addition of radioactive phage was preceded by sufficient nonradioactive phage to infect most of the cells in the culture, the amount of breakdown of labelled virus increased to about 55% within 20 min. at which time breakdown practically ceased. Control experiments indicated that dead phage in the labelled preparations contributed little to the acid soluble P^{32} fraction in these experiments and that the breakdown resulted from infection of the cells by virus. It is suggested that infection of cells by phage stimulates some mechanism whereby phage adsorbed to the cell at a later time is broken down extensively at the cell surface. The experimental results are interpreted in the light of this concept.

Introduction

The purpose of this work was to investigate some of the phenomena connected with the apparent disappearance of the virus particle following infection of its host. That some extensive alteration does take place in virus structure shortly after infection has already been suggested by many workers. Thus studies by Hoyle (8) and by Henle *et al.* (7) on influenza virus indicated that once adsorbed to the host cells the virus could not be recovered by rupture of such cells until several hours after infection. Similar behavior of other viruses such as those of vaccinia, ectromelia, and psittacosis has led to the suggestion by Bauer (1) that the most probable change in the virus seems to be "the dissolution of the virus body into a soluble phase which then diffuses through a considerable volume of cytoplasm and organizes it into virus material by an action upon the chromidia and their enzymes".

Certain bacteriophages appear to undergo a somewhat analogous change. By breaking open the cell at different times after the infection of *Escherichia coli* with some phages of the T group, Doermann (6) has demonstrated that no virus could be detected until about the middle of the "latent period". In a recent review Luria (13) has correlated this finding with those obtained

[1] *Manuscript received January 16, 1951.*

Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont.

Aided by a grant from the National Cancer Institute of Canada.

in genetic and other studies on bacteriophage infection. He has suggested that infection produces a reorganization of the cell, the virus particle being divided into a number of genetic units. Following replication of these units they are reorganized into mature phage particles. Direct evidence that some physical breakdown of the infecting virus particle does occur is given by Putnam and Kozloff (14). These workers found that on lysis of cells infected with P^{32} labelled T6r^+ bacteriophage the greater part of the isotope was found in the medium as low molecular weight phosphorus. A significant amount of the isotope appeared in the newly formed virus.

While the explanation advanced by Luria (13) to account for the apparent disappearance of the infecting bacteriophage is an attractive one, it is based to a large extent on indirect evidence. In an attempt to throw more light on the mechanism of infection we have carried out experiments with T2r^+ bacteriophage labelled with P^{32}. Preliminary work revealed that shortly after infection of the cells P^{32} appeared in the medium in a form soluble in 5% trichloroacetic acid. This suggested a rapid breakdown of the infecting virus. Thus, using the formation of acid soluble P^{32} as a measure of breakdown, the changes occurring in cells infected under different experimental conditions were investigated. The results which have been summarized in a preliminary paper (10) are now described in detail.

Materials and Methods

Bacteriophage Preparations

The growth, purification, and properties of T2r^+ bacteriophage labelled with P^{32} have been reported in detail (11). Purified phage was kept at 5° C. as a suspension in 0.85% sodium chloride solutions (saline) usually at a concentration of 1 to 3 $\times$ 10^{11} phage per ml. Before use in the metabolism experiments described below, the required amount of saline suspension was diluted to about 10 ml. with tryptose broth and kept at room temperature for at least one hour. As described previously (11) this procedure facilitated dispersal of the virus particles and yielded a larger number of plaque forming units. Estimations of virus concentrations, phage per ml., were carried out using the plaque counting method. In the experiments described here the plating efficiency has not been utilized in calculating the ratios of phage particles per cell and the number of phage particles was calculated directly from the plaque count assay.

General Procedure Utilized in Metabolism Experiments

In carrying out experiments on the breakdown of T2r^+ bacteriophage after infection of the host cells, the general procedure used in the present work was as follows. A quantity of tryptose broth, usually 150 ml., was inoculated with *E. coli* B from an 18 hr. slope to give a concentration of approximately 10^7 cells per ml. The culture was aerated at 37° C. until the concentration of cells

reached 2 to 7 $\times$ 10^8 per ml., the density being estimated spectrophotometrically as previously described (11). After the culture was chilled, it was centrifuged at 4300 *g* at 5° C. in an angle centrifuge to sediment the cells. The cells were then resuspended in tryptose broth, previously warmed to 37° C., to give the required volume containing 2 $\times$ 10^8 cells per ml., and the suspension was placed in a large pyrex tube equipped with a stopcock to facilitate the removal of samples. To this cell suspension was added a predetermined amount of labelled bacteriophage and aeration of the culture was begun immediately at 37° C. At intervals 14.8 ml. aliquots were transferred to chilled tubes graduated at 15 ml. and containing 0.2 ml. of a dialyzed 1% solution of crystalline egg albumin to act as a protein carrier. Two and one-tenth ml. of 40% trichloroacetic acid (TCA) was then added to give a final concentration of 5% TCA and the tubes were kept in an ice bath for at least 15 min. The precipitate was removed by centrifuging at 4300 *g* for 15 min. in an angle centrifuge at 5° C. and the supernatant carefully decanted off. Radioactivity estimations on these supernatants were carried out using a liquid counter, 10 ml. capacity, of the type described by Veall (16), in conjunction with a conventional scaling unit.

The P^{32} present in the TCA supernatant solution was presumed to be released during the metabolism of the labelled bacteriophage in the infected cells and, calculated as a percentage of the amount of P^{32} originally added in the radioactive phage, was used as a measure of breakdown for the infecting virus. In most experiments utilizing this technique sufficient samples were taken to permit the breakdown of phage to be followed from the time of addition of the radioactive phage until lysis of the culture. Most of the experiments described were carried out with seven different preparations of purified radioactive virus 7, 8, 9, 9A, 11, 14, and 15.

Experimental

Breakdown of P^{32} Labelled Bacteriophage with Single Infection of the Host cells

Experiments were carried out with each of seven different preparations of labelled virus to determine the extent of breakdown when cells, at a concentration of 2 $\times$ 10^8 per ml., were infected with phage in the ratio of one phage per five cells. The first sample was taken immediately following the addition of phage after which the culture was mixed continuously with a stream of sterile air; thereafter samples were removed at closely spaced intervals, treated as described above, and the percentage TCA soluble P^{32} was determined for each.

In Fig. 1 the percentage TCA soluble P^{32} is plotted against time in minutes for three experiments carried out on different days with one radioactive virus preparation (No. 8).The last point on Curve 2 represents a sample taken immediately after lysis of the culture. It is observed that the curves are two-step in shape. Since the latent period of this phage is about 22 min. the second rise began shortly after the time when the first release of newly

formed virus would be expected from the infected cells. In several experiments of this type titrations carried out at intervals indicated an increase in phage beginning at about 25 min.

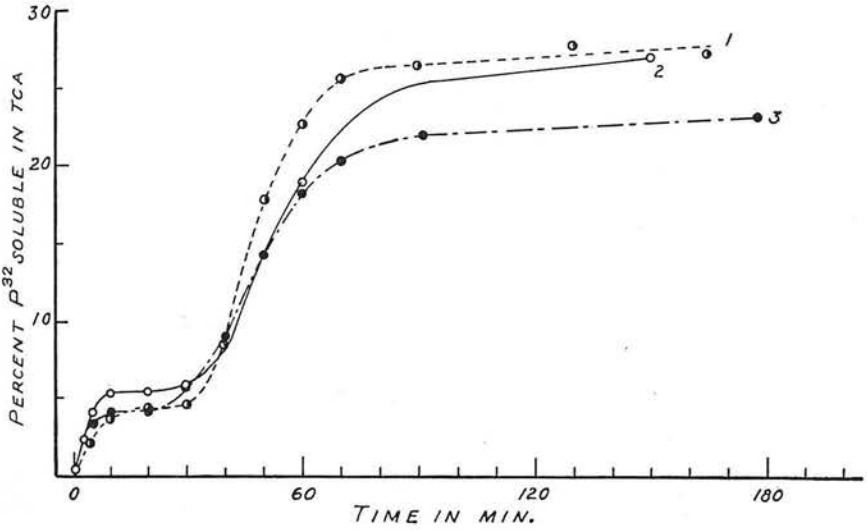

FIG. 1. Release of TCA soluble P^{32} when *E. coli* at 2×10^8 cells per ml. was infected with 0.2 labelled phage per cell. The experiments were performed with one radioactive virus preparation (No. 8). The last point on Curve 2 represents a sample taken after lysis of the culture.

While the height of the first step of the curve indicated 4 to 5% breakdown of the radioactive phage in the first 20 min. or so, the number of counts per minute (c.p.m.) in these samples was only 5 to 10 c.p.m. over the background of the counter (10 c.p.m.). Such a step in the curve was observed consistently in experiments of this type but because of the small increases in radioactivity further experiments were designed to seek more convincing proof of the effect. Consequently, tests were carried out in which the concentration of cells was increased to 6×10^8 per ml., and radioactive phage was added at a ratio of one phage to five cells. Curves of similar shape to those in Fig. 1 were obtained, the first step indicating 3 to 5% breakdown of the radioactive phage. Alteration in the ratio of infection from 0.15 to 0.33 phage per cell, while the cell concentration was retained constant at 6×10^8 per ml., did not alter the height of the first step. These results were interpreted as strong evidence that the first step represented a real breakdown of the phage particles.

Similar results were observed with radioactive virus preparations 7, 9, 9A, 11, 14, and 15. The heights of the first steps in the respective curves represented 3.0, 4.5, 3.5, 4.0, 3.4, 7.0% breakdown and the final amounts of breakdown at lysis of the cultures were 42, 27, 36, 24, 24, 33% respectively. Thus the curves for all seven phage preparations were two-step in shape but considerable variation was observed in the final amount of breakdown undergone by the virus. Time of lysis in these cultures varied between 115 to

310 min. following infection of the cells. There appeared to be no additional release of TCA soluble P^{32} on lysis, since, in several experiments, samples taken some time before lysis and immediately after lysis showed the same percentage breakdown of the virus.

Breakdown of Bacteriophage with Multiple Infection of the Cells

Fig. 2 illustrates the formation of 5% TCA soluble P^{32} in the culture when *E. coli*, at a concentration of 2×10^8 cells per ml., was infected with radioactive phage (Preparation 8) in ratios of 3, 5, and 10 virus per cell. The last

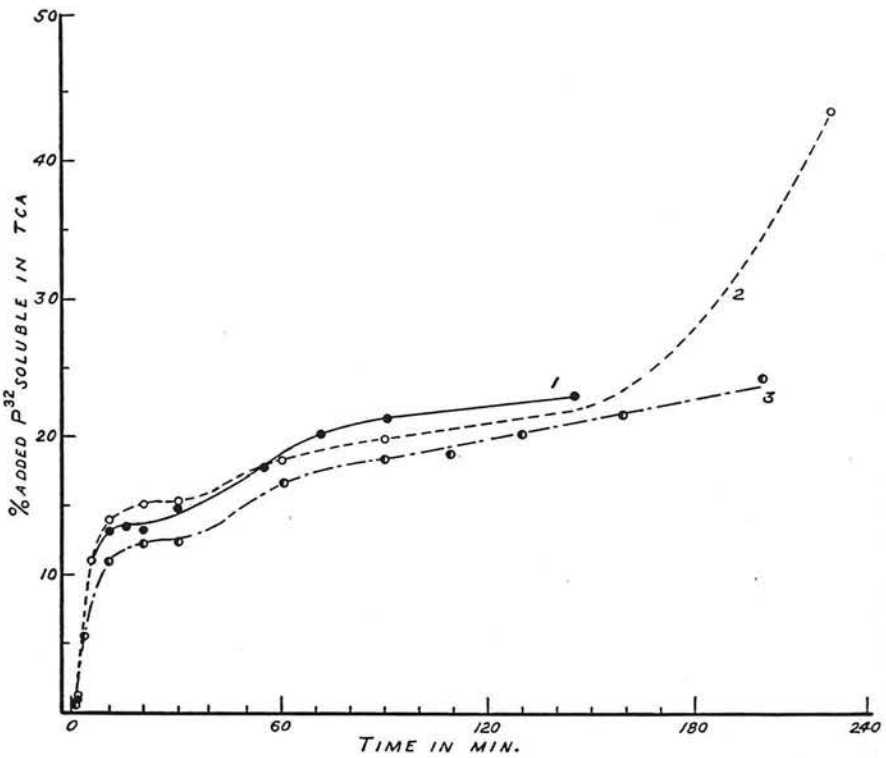

FIG. 2. Relationship between multiplicity of infection with radioactive phage and release of TCA soluble P^{32}. Curves 1, 2, and 3 represent infecting ratios of 3, 10, and 5 phage per cell respectively. Cell concentration was 2×10^8 per ml. in each case.

points on Curves 1 and 3 represent samples taken immediately after lysis of the cells. In a similar set of experiments with Preparation 14A, labelled phage was added to the cells in the ratios of 3, 5, 10, and 15 phage per cell. At 90 min. after infection the breakdown of virus was 21, 21, 25, and 19% respectively. Similar curves were obtained with phage preparations, 7, 9, 9A, and 11 at infecting ratios of three to five phage per cell. For these preparations the TCA soluble P^{32} released at 90 min. after infection was 31, 24, 23, and 24% respectively. Experiments with infecting ratios of 20 to 25 phage per

cell gave variable results. In one instance the breakdown was 25% while two other experiments gave 12 and 13% breakdown. In the latter two experiments lysis of the culture was considerably advanced 30 min. after addition of the phage. In a single experiment in which an attempt was made to follow the breakdown with an infecting ratio of 50 phage per cell the culture lysed completely in less than 20 min. It is probable that this was a case of "lysis from without" as described by Delbrück (5). That the course of the reaction was different from that in the usual multiple infection type of experiment is indicated by the finding that only 2.4% of the P^{32} was present in TCA soluble form in the lysed culture.

Estimations of the amount of phage adsorption were not carried out in most of the experiments of the multiple infection type described above. However, where infectivity titration of the unadsorbed virus was made, the amount of phage adsorbed was 95% or greater 10 min. after addition of phage at ratios up to 10 particles per cell.

While most experiments carried out with multiple infection of cells by radioactive phage conformed to the general picture represented by Fig. 2, certain irregularities were observed from time to time which should be mentioned here. Often there was pronounced flattening of the breakdown curves between 15 to 30 min. following infection of the cells. This was followed by a small increase up to 60 to 70 min. before the curves flattened off and maintained a constant slight slope to the time axis. This effect is well demonstrated in the curves of Fig. 2. However, in a number of experiments of this type the small step between 15 to 30 min. was absent, the curves rising smoothly and flattening off at 20 to 30 min. without any apparent inflection. In many experiments lysis occurred at 150 to 180 min. following multiple infection of the cells. However, there was very great variation in lysis time, some cultures lysing in 90 min. while others took up to 250 min. to lyse. While numerous experiments were carried out to investigate this observation we have been unable to explain the variability. The course of the breakdown reaction with labelled phage did not seem to be influenced by these large variations in lysis time although on isolated occasions, such as represented by Curve 2, Fig. 2, there was a second large rise in the breakdown curve before lysis. This behavior was observed in three experiments for cultures that took about four hours to lyse. In many other similar experiments with lysis periods of four hours this second rise was not observed.

Breakdown of Labelled Bacteriophage When Added at an Interval Following Infection with Nonradioactive Phage

Effect of Variations in the Amount of Normal Phage

When purified nonradioactive bacteriophage ("normal" phage) was added to a cell culture containing 2×10^8 cells per ml. in a ratio of 10 virus particles per cell (primary infection) and, after a five minute interval, radioactive phage was added in the ratio of two phage per cell (secondary infection), the

breakdown of radioactive virus was as shown in Curve 1, Fig. 3. Primary infection with one normal phage per cell gave Curve 2 which is extrapolated to lysis time, while Curve 3 was obtained with a primary infection of one phage

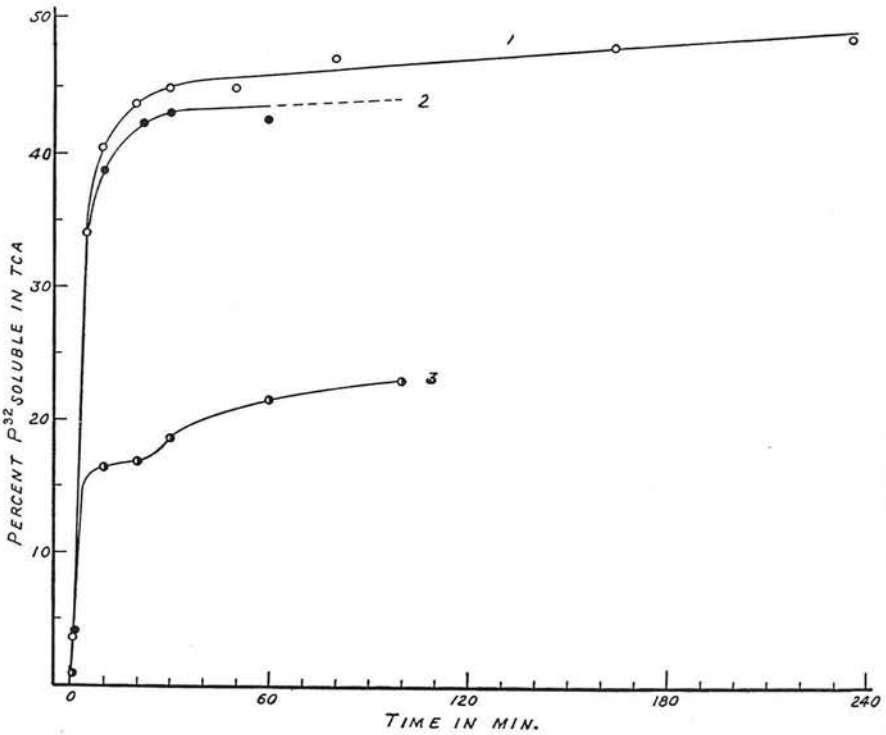

FIG. 3. Effect of variation in primary infecting ratio of nonradioactive phage on breakdown of labelled phage added five minutes later. Primary infecting ratios 10, 1, and 0.15 phage per cell for Curves 1, 2, and 3 respectively at cell concentration of 2×10^8 per ml. The time axis starts with the addition of radioactive phage.

per 6.6 cells. In both these experiments secondary infection was with two radioactive phase (Preparation 8) per cell five minutes after primary infection. In the above mentioned experiment in which one phase per cell was used for primary infection, the actual infecting ratio was probably closer to 1.5 if the plating efficiency of the phase titration is taken into account. Assuming a Poisson distribution some 80% of the cells would be infected. In Fig. 3, the time axis starts at the addition of radioactive phase.

These and other experiments demonstrated that if sufficient normal phase was added to infect most of the cells in a culture, radioactive phase added after an interval of five minutes was broken down rapidly to the extent of 50 to 55%. There was no significant difference when the primary infection was varied between 1 and 10 normal particles per cell. However, when the primary infection was one normal phase per 6.6 cells the percentage breakdown of radioactive virus added after five minutes was very much less. In

most of the experiments of this type the normal phage used for primary infection was taken from suspensions of purified virus. When phage from a freshly lysed culture was used for primary infection a similar picture was obtained. To exclude the possibility that some factor in the lysate, other than phage, might be partially responsible for the extensive breakdown of secondarily adsorbed phage, the following experiment was performed.

More than 99% of the virus was sedimented from a fresh lysate of T2r^+ phage by centrifugation at 120,000 *g* for 60 min. Three ml. of the supernatant solution containing 9×10^7 phage per ml. was added to 150 ml. of a culture of 2×10^8 cells per ml. and after 12 min. three labelled phage per cell were added. The resulting breakdown curve was similar to those obtained with multiple infection of the cells by radioactive virus such as are shown in Fig. 2. This is considered as additional evidence that the increased breakdown of radioactive phage caused by a preliminary infection with normal phage is due to some property of the phage.

Effect of Varying the Interval Between Primary Infection with Normal Phage and Secondary Infection with Labelled Phage

In a series of experiments cell suspensions at a concentration of 2×10^8 cells per ml. were infected with 10 normal phage particles per cell. After an interval two labelled phage (Preparation 8) per cell were added, the interval being varied from 5, 15, 45, and 155 min. from one experiment to another. Within experimental error the curves were the same as Curve 1, Fig. 3. The amounts of breakdown at 60 min. after secondary infection were 46, 41, and 43% for the experiments with 5, 15, and 45 min. intervals. In the experiment with 155 min. between primary and secondary infection lysis occurred 25 min. after the addition of radioactive phage; the amount of breakdown at lysis was 47.5%. When the interval was reduced to one minute the shape of the breakdown curve was similar to Curve 1, Fig. 3, but the total amount of breakdown was much less, amounting to 33% 60 min. after infection.

Effect of Varying the Ratio of Secondary Infection with Radioactive Phage

In a further series of experiments cultures containing 2×10^8 cells per ml. were infected with three normal phage per cell. After an interval of five minutes radioactive phage (Preparation 9) was added at ratios of 0.20, 5, 30, and 60 phage per cell. The shapes of the breakdown curves were similar to that of Curve 1, Fig. 3. Sixty minutes after secondary infection the breakdown was 57, 53, 59, and 58% for the respective infecting ratios. It seems probable from these results that each of the phage particles in the secondary infection was broken down to the increased extent.

Many similar experiments were carried out with other radioactive phage preparations. With primary infecting ratios of 3 to 10 phage per cell, an interval of five minutes, and secondary ratios of 2 to 10 radioactive phage per cell, the curves were invariably similar in shape to Curve 1, Fig. 3. At 60 min. after secondary infection the amount of breakdown of labelled phage

was 56, 46, 59, and 45% for Preparations 7, 8, 9A, and 11. Some preliminary experiments have been performed with T2r^+ phage which was labelled with P^{32} in the synthetic "F" medium described by Cohen and Anderson (4). This purified phage when used to infect cells in broth gave similar results.

As in the experiments on multiple infection described in a previous section, there was a large variation in lysis time in the experiments just described. Lysis usually occurred 160 to 190 min. after primary infection but on occasions occurred as early as 90 and as late as 280 min. There appeared to be no correlation between lysis time and multiplicity of infection of the cells. Considerable variation in lysis time, however, apparently had little influence on the course of the breakdown of infecting phage.

Breakdown of Radioactive Phage When Added at Successive Intervals to a Single Culture of Cells Infected with Normal Phage

Experiments as described above indicated that when cells were multiply infected with radioactive phage on the average about 23% of the P^{32} was broken down to a form soluble in 5% TCA. However when the cells had been previously infected with normal phage the amount of breakdown was increased to about 55%. It was therefore of interest to determine whether several quantities of radioactive phage added at successive intervals to a cell suspension, previously infected with normal phage, would each break down to this increased extent. Such an experiment was carried out as follows.

Normal phage was added to a culture containing 2×10^8 cells per ml. in the ratio of three phage per cell. After five minutes three radioactive phage (Preparation 9) per cell were added and the culture was aerated at 37° C. Equal quantities of labelled virus were added at 23 and 43 min. after addition of the first radioactive phage. Samples were withdrawn from the culture at intervals, and the per cent breakdown determined for each quantity of radioactive phage added; the results are shown in Fig. 4. Each of three quantities of radioactive phage was extensively broken down, the amount of breakdown being about 56, 53, and 74% respectively.

Distribution of TCA Soluble P^{32} Between Cells Infected with Labelled Phage and Supporting Medium

The following experiment was performed to determine whether TCA soluble P^{32} released by the infecting labelled virus was liberated rapidly into the medium or remained in the cell. Information was also obtained on the completeness of extraction of TCA soluble P^{32} by the technique utilized in the metabolism experiments described in preceding sections.

A culture containing 2×10^8 cells per ml. was infected with three normal phage per cell and five minutes later seven radioactive phage (Preparation 11) per cell were added. The culture was aerated at 37° C., and 35 ml. samples were withdrawn 11 and 60 min. after the secondary infection. These were immediately chilled in an ice bath and centrifuged at 5° C. The sedimented cells were resuspended in 35 ml. 5% TCA and allowed to stand in an ice bath

to allow extraction of P^{32} by the TCA. Aliquots were removed after 15 and 75 min. and centrifuged, P^{32} estimations being carried out on the resulting supernatants. Two 14.8 ml. aliquots of the supernatant solution resulting

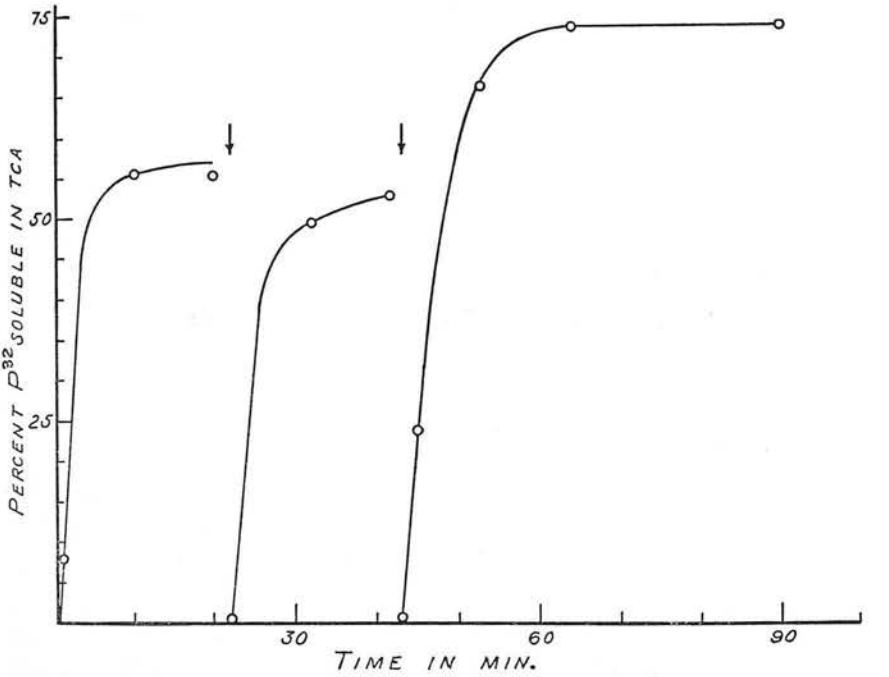

FIG. 4. Breakdown of three quantities of radioactive phage added 5, 27, and 48 min. after infection of 2×10^8 cells per ml. with three nonradioactive phage per cell. Three labelled phage per cell were added in each case. Time axis starts from first addition of radioactive phage and time of other additions represented by small arrows.

from the removal of the infected cells were taken. To the first was added 0.2 ml. albumin and 2.1 ml. 40% TCA in the cold. After this mixture had been allowed to stand 15 min. in an ice bath it was centrifuged and P^{32} was determined in the supernatant. P^{32} was determined in the second aliquot after the addition of 0.2 ml. albumin and 2.1 ml. in water. The results are presented in Table I.

The total TCA soluble P^{32} is obtained by adding the figures in Columns 2 and 4. This amounts to 41% and 47% of the P^{32} added in labelled phage for the 11 and 60 min. samples respectively. It is observed that 12 and 3% of the TCA soluble phosphorus remained in the cells at 11 and 60 min., indicating that the TCA soluble P^{32} released from the infecting phage passed rapidly into the medium. Also, 15 min. treatment with TCA was sufficient to remove whatever soluble P^{32} did remain in the cells. The figures in Column 5 represent TCA soluble P^{32}, P^{32} contained in unadsorbed phage and possibly some breakdown products of intermediate size, while those in Column 4 the corresponding

TABLE I

DISTRIBUTION OF P^{32} SOLUBLE IN 5% TRICHLOROACETIC ACID BETWEEN CELLS AND MEDIUM AT TWO INTERVALS AFTER SECONDARY INFECTION WITH SEVEN RADIOACTIVE VIRUS PARTICLES PER CELL. PRIMARY INFECTION WITH THREE NORMAL VIRUS PER CELL FIVE MINUTES BEFORE ADDITION OF RADIOACTIVE PHAGE*

Time of sample, min.	Sedimented infected cells		Supernatant from infected cells	
	Suspended in 5% TCA 15 min.; P^{32} in extract, c.p.m.**	Suspended in 5% TCA 75 min.; P^{32} in extract, c.p.m.	Treated with 5% TCA; P^{32} in resulting supernatant, c.p.m.	No further treatment; P^{32}, c.p.m.
11	62	59	456	745
60	19	20	569	828

* *Amount of P^{32} added in labelled phage 1257 c.p.m. calculated to same basis as figures in the table.*

** *c.p.m. = counts per minute.*

amounts of TCA soluble P^{32}. From these results it was calculated that the radioactive phage was adsorbed to the cells to the extent of at least 77 and 79% at 11 and 60 min. Another such experiment gave similar results.

Control Experiments

The possibility was envisaged that the breakdown of radioactive phage reported in previous sections might be partially due to noninfective, "dead", phage present in the purified preparations. In view of our earlier work (11) it was felt that the preparations of purified radioactive phage consisted essentially of infective particles. Nevertheless, if only a few per cent of the phage particles were dead, and were broken down completely in the metabolism experiments, the results might be difficult to interpret. It was also possible that a small proportion of dead cells might influence the breakdown of the infecting phage.

Consequently, a number of experiments were performed with dead radioactive phage and dead cells, the percentage breakdown of the phage being measured by the same techniques used in the previous experiments. The cells and phage were killed by heating for 45 min. at 70° C. Thermal inactivation, rather than inactivation with ultraviolet radiation, was chosen since it was felt that this approached more closely the method by which phage and cells would be killed during their routine preparation. The results obtained in these experiments are shown in Table II.

It is observed that in none of these control experiments was there any significant breakdown of dead radioactive phage during the first 60 min. except in the case of dead phage added to a lysate freshly prepared by the action of T2r^+ phage on *E. coli*. Presumably the heat inactivated phage was

TABLE II

CONTROL EXPERIMENTS WITH HEAT KILLED CELLS AND LABELLED BACTERIOPHAGE

Type of experiment	Per cent breakdown of radioactive phage
Dead phage, dead cells	0.6% at zero time, 2.2% at 60 min., 3.8% at 97 min.
Dead phage, live cells	1.0% at zero time, 1.0% at 30 min., 0.8% at 105 min.
Live phage, dead cells	0.4% at zero time, 2.9% at 70 min., 7.8% at 101 min.
Live phage in fresh lysate of T2r^+	0.4% at zero time, 0.9% at 70 min., 5.9% at 142 min.
Dead phage in fresh lysate	{0% at zero time, 14% at 28 min., 34.2% at 120 min. {0.2% at zero time, 18.2% at 120 min.
Dead phage added to live cells five minutes after addition of three normal phage per cell	0.5% at zero time, 0.5% at 30 min., 0.9% at 120 min.

digested by the desoxyribonuclease (DNase) reported to be present in fresh lysates (2). It is worth noting that, after 10 min., there was no adsorption of the heat killed bacteriophage to either live or dead cells.

In the experiments on single infection of the cells with labelled phage, evidence has been presented that the initial step in the curve represented a real and significant breakdown of the virus to the extent of about 5%. It still seemed possible, nevertheless, that this breakdown might result from the destruction of a small amount of radioactive pentose- or desoxypentosenucleic acid present in the virus preparation as an impurity, rather than a metabolic rearrangement of the structural constituents of the virus. To gain information on this point experiments were carried out in which purified labelled phage preparations were subjected to the action of ribo- and desoxyribonuclease.*

In a typical experiment the amount of TCA soluble P^{32} in the purified virus was 0.5%. This amount was not increased by incubation with crystalline ribonuclease (RNase) at 37° C. for 30 min., while the TCA soluble P^{32} increased to 1.73% upon incubation with DNase for 30 min. Neither enzyme reduced the infectivity of the phage under the above conditions. Upon heating the phage for 15 min. to 60° C. the infectivity was reduced to 50% and after 60 min. at 70° C. the infectivity was less than 1% of the original. When DNase was allowed to act on these heated preparations 20.8 and 89.5% respectively of the P^{32} was converted to a TCA soluble form. In neither case did RNase liberate any TCA soluble P^{32}. There was thus a rough correlation between the amount of inactivation of the phage and the amount of acid soluble P^{32} formed by action of DNase on it. This finding was consistent with the previous observation that a considerable portion of the P^{32} was converted to an acid soluble form when heat killed labelled phage was aerated in a fresh T2r^+ broth lysate.

* *We are indebted to Dr. G. C. Butler for a specimen of highly purified desoxyribonuclease.*

From these results it appeared probable that the small amount of acid soluble P^{32} formed by the action of DNase on freshly purified phage arose from a small amount of dead phage in these preparations. Next, an effort was made to determine whether labelled phage subjected to the action of DNase would behave in the same manner as an untreated preparation upon infection of the host cells.

Accordingly, a freshly purified preparation of labelled phage was divided into two portions. One of these was treated with highly active DNase for 30 min. at 37° C. This procedure increased the proportion of TCA soluble P^{32} from 0.4 to 1.98%. The treated and untreated phage were then used to infect two cultures of *E. coli* containing 2×10^8 cells per ml. in the ratio of one virus to five cells, and the cultures were aerated at 37° C. Samples were taken at intervals for determination of TCA soluble P^{32}. When allowance was made for the acid soluble P^{32} which had been liberated by the enzyme, the two breakdown curves were the same within experimental error. Thus preliminary treatment with DNase did not influence its breakdown when cells were singly infected with the virus.

Discussion

Previous work by Cohen (3) and Lesley *et al.* (11) with T2r^+ phage and Kozloff and Putnam (9) with T6r^+ phage has indicated that the P^{32} in labelled virus is almost exclusively contained in the desoxypentose nucleic acid moiety and is incorporated into its structure. When the host cell was infected with labelled virus a certain portion of the P^{32} changed to a form soluble in 5% trichloroacetic acid. Since the infecting virus generally contained less than 0.5% of its P^{32} in a TCA soluble form, it seems reasonable to assume that the formation of TCA soluble P^{32} during the course of infection did represent degradation of the virus nucleic acid. Live radioactive phage did not break down to any appreciable extent when added to a fresh lysate. Therefore an extracellular breakdown mechanism, such as the liberation of an autolytic enzyme into the medium, seems to be excluded. The control experiments further indicated that the formation of acid soluble P^{32} resulted largely from the degradation of fully infective virus rather than from the breakdown of a portion of dead phage in the purified preparations.

That the breakdown may be extensive is indicated by the observation of Putnam and Kozloff (14) that on lysis of cells infected with labelled T6r^+ phage about 50% of the TCA soluble P^{32} was inorganic phosphate. Using the formation of TCA soluble P^{32} during infection as a criterion, the breakdown of infecting phage proceeded in different ways depending on the method of infecting the cells.

It would appear that the first 5% step in the single infection curve represents the breakdown of invading phage under the simplest set of conditions. A reasonable explanation of most of our results can be devised if it is then assumed that infection of the cells with phage stimulates the formation of

some mechanism in the infected cell which breaks down phage particles arriving at a later time to the extent of about 55%. For the purpose of discussion this phenomenon will be called the "stimulation effect", and the initial infection with normal phage will be said to "stimulate" the cells. The stimulation is produced rapidly after adsorption of phage. Radioactive phage added one minute after primary infection was broken down by about 33% indicating that the effect was already apparent; the maximum effect was observed within five minutes of primary infection and was probably achieved within two to three minutes. Once cells were stimulated their capacity for breaking down phage remained undiminished until at least 25 min. before lysis; possibly the stimulation remains until immediately before lysis.

It is postulated that the stimulation effect may be a surface phenomenon and that phage adsorbed to the cell before it is produced are inaccessible to later breakdown by it, perhaps owing to their penetration of the cell. A tentative explanation of the multiple infection curve can then be made as follows. When phage is added to cells to give multiple infection, the greater portion is adsorbed in the first two minutes. It is suggested that these phage are broken down by about 5%. In the process the stimulation effect is initiated and phage adsorbed after more than two minutes suffers the full stimulation breakdown of 55%. The curve is thus the resultant of two processes. Let us consider two experiments with a labelled phage preparation in one of which the infecting ratio is 10 and in the other 100 phage per cell, the cell concentration being held constant at 2×10^8 per ml. Under these conditions about 75% of the phage is adsorbed in two minutes in each instance. Assume that this phage is broken down by 5% and the remainder, adsorbed at a later time, is broken down by 55%. In each case it may be calculated that the maximum amount of breakdown is 17.5%. This theoretical computation thus shows that the breakdown curves for widely differing multiplicities of infection should achieve the same maximum value, which, in fact, is close to the determined average value of about 23% breakdown for this type of experiment. The difference between theoretical and experimental values may result from the assumption that stimulation does not begin until two minutes after mixing phage and cells. In practice stimulation breakdown is apparent after one minute and the calculated figure should therefore be a minimum value.

The small plateau often found in the experimental multiple infection curves between 15 and 30 min., and the extensive rise found on isolated occasions before visible clearing of the culture, are probably due to lysis of some cells. Readsorption of the progeny on other infected, and consequently stimulated cells results in release of TCA soluble P^{32} from the isotope contained in the progeny and therefore a rise in the curve.

When cells are singly infected with labelled phage about 5% of the P^{32} appears in an acid soluble form. Whether this is removed from the phage particle after adsorption and before entry into the cell or whether it is released by an intracellular reaction is unknown. It is assumed that a considerable

portion of the remaining P^{32} is transferred to the first generation progeny. At the end of the 21 min. latent period the progeny is released into the medium and rapidly infects the remaining cells with, on the average, about 15 phage per cell under the conditions used in the present experiments. The second step is then a multiple infection breakdown curve of the type discussed above. Assuming that all the first generation phage is broken down by 55% by the stimulation effect during the second cycle of infection, a transfer to the progeny of 50% of the P^{32} in the original labelled phage would be necessary during the single infection cycle to account for the height of the second step in the breakdown during the second cycle.

It has been demonstrated by Putnam and Kozloff (14) that, when cells are multiply infected with labelled T6r^+ phage, 22 to 42% of the P^{32} appears in the progeny. Our own results (unpublished) show a transfer of 15 to 25% P^{32} to progeny in experiments of a similar type with T2r^+ phage. There is, consequently, a considerable discrepancy between the amount of P^{32} transferred to progeny in experiments of the *multiple* infection type, and the amount which should, according to the above explanation, be transferred during *single* infection to account for the second step in the breakdown curve.

This discrepancy may be resolved in either of two ways. Firstly, the explanation given above for the second step may be incorrect and the rise could be accounted for by release of acid solution P^{32} upon lysis of the singly infected cells. It is felt that this is unlikely since, during the large number of experiments carried out in the course of this work, visible clearing of a culture has not been observed to release additional TCA soluble P^{32}. Secondly, the proportion of P^{32} in the infecting phage which is available for incorporation into progeny in the multiple infection type of experiment is reduced by the stimulation breakdown reaction. The stimulation breakdown does not enter the picture during the first cycle of single infection, and it is thus possible that the proportion of P^{32} transferred to progeny in this cycle is a good deal higher than in multiple infection. One possible means of settling this point would be to determine the transfer of P^{32} to progeny for a virus which shows no stimulation effect, and preliminary experiments along these lines have suggested that T^1 or T^7 coliphages may be suitable.

The mechanism of the stimulation effect and of phage breakdown by it are unknown but the process bears some resemblance to fertilization of the ovum by a sperm. Apparently a single sperm penetrates the egg and some mechanism is set in operation which bars the entry of further sperms (12, 15). It may be that infection of the cell by phage induces the rapid formation of a nuclease or protease system at the surface and as more phage are attached to the cell they are digested. Up to the present, this breakdown reaction has been investigated using as an arbitrary measure the formation of TCA soluble P^{32}. However, it seems plausible to assume that acid soluble P^{32} is not the only product of the reaction and that other fragments of phage breakdown which are not soluble in TCA are also present. Investigation of these products may give some clue to the mechanism of the reaction.

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STUDIES ON THE RELATIONSHIP BETWEEN VIRUS AND HOST CELL

III. THE BREAKDOWN OF P^{32} LABELLED T2r^+ BACTERIOPHAGE ADSORBED TO *E. COLI* PREVIOUSLY INFECTED BY OTHER COLIPHAGES OF THE T GROUP

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Reprinted from Canadian Journal of Medical Sciences, 29 : 144-148. June, 1951

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III. THE BREAKDOWN OF P^{32} LABELLED T2r^+ BACTERIOPHAGE ADSORBED TO *E. COLI* PREVIOUSLY INFECTED BY OTHER COLIPHAGES OF THE T GROUP[1]

BY R. C. FRENCH, S. M. LESLEY, A. F. GRAHAM, AND C. E. VAN ROOYEN

Abstract

Infection of *E. coli* with T2*r*, T4r^+, T4*r*, T5, T6r^+, or T6*r* phages induces the formation of a mechanism which extensively degrades P^{32} labelled T2r^+ phage adsorbed to the cell shortly afterwards, about 50% of the P^{32} being converted to a form soluble in 5% trichloroacetic acid. Each of the above viruses is as efficient in this respect as a preliminary infection with T2r^+ phage itself. Previous infection of the cells with T1, T3, or T7 phages does not stimulate this mechanism to break down labelled T2r^+ virus. When T1, T3, or T7 phage, then T2r^+ phage, and finally P^{32} labelled T2r^+ phage were added to cells, with an interval of several minutes between each addition, the results indicated that adsorption of T2r^+ to the cell was not sufficient *per se* to stimulate the breakdown of labelled phage. Apparently actual infection of the cell by T2r^+ virus was required before the breakdown mechanism was induced.

Introduction

In previous papers (6, 8) it was reported that infection of *E. coli* by T2r^+ bacteriophage labelled with P^{32} resulted in the rapid appearance of acid soluble P^{32} in the culture. This low molecular weight P^{32} was considered to result from a chemical breakdown of the virus nucleic acid during, or shortly after, its adsorption to the host, and was used as a measure of the process.

When the labelled phage was added to a cell culture in an amount sufficient to ensure that, of the cells infected, practically all had adsorbed only one virus particle, the breakdown amounted to about 5%. However, when the cells were first infected with purified nonradioactive T2r^+ phage and this was followed five minutes later with labelled virus, the latter was rapidly broken down by about 55%. These results led to the suggestion that infection of the host cell with T2r^+ phage stimulated the rapid formation of some mechanism in the cell which extensively degraded other T2r^+ particles adsorbed to the infected cell at a later time.

In order to gain more information about this "stimulation effect" it was of interest to determine whether other phages would stimulate the cells to break down T2r^+ phage in a similar manner. Accordingly, experiments were performed in which P^{32} labelled T2r^+ phage was added to cells previously infected with other coliphages of the T group (1), and the extent of the breakdown of the labelled phage was determined. The results are reported in the present paper.

[1] *Manuscript received February 12, 1951.*

Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont.

Aided by a grant from the National Cancer Institute of Canada.

Material and Methods

T2r^+ bacteriophage was labelled with P^{32} and purified as described previously (7).

The general method of carrying out the experiments was as follows. Bacto-tryptose broth was lightly inoculated with *E. coli* from an 18 hr. slope and aerated at 37° C. until the cells reached a concentration of 3 to 6 $\times$ 10^8 per ml. After sedimenting the cells they were resuspended in the required volume of fresh warm broth at a concentration of 2 $\times$ 10^8 cells per ml. The cells were then infected by adding a large excess of one of the T coliphages and after a predetermined time labelled T2r^+ phage was added. Aliquots were removed from the culture at intervals and the amounts of P^{32} soluble in 5% trichloro-acetic acid were determined as described in detail elsewhere (8). The acid soluble P^{32} was calculated as a percentage of the amount of P^{32} added with the labelled virus and thus used as a measure of breakdown of T2r^+ phage.

Experimental

The Breakdown of Labelled T2r^+ Phage Added to Cells Infected with Other T Coliphages

In a series of experiments cultures of *E. coli* were infected with each of the seven coliphages of the T group, and also with the fast lysing *r* (4, 5) mutants of phages T2, T4, and T6 (primary infection). Sufficient excess of virus was added to ensure that practically all the cells would be rapidly infected. After an interval labelled T2r^+ phage was added to the cultures (secondary infection) and a sufficient number of samples were taken to determine the course of the breakdown. The results shown in Table I represent the maximum breakdown for each experiment.

It is observed that the 10 phages used for primary infection fall into two distinct groups, T1, T3, and T7 do not stimulate the cells to break down T2r^+ phage, whilst all the other phages do induce the stimulation effect just as efficiently as a primary infection with T2r^+ phage itself.

Some variation in results was observed when primary infection of the cells was accomplished with T1, the amount of breakdown of secondarily adsorbed T2r^+ being as high as 13.5% and as low as 3.5%. There was generally a partial visible clearing of the culture 15 to 20 min. after infection with T1, corresponding roughly to the end of the latent period of this virus, but lysis of the culture seldom proceeded to completion. It seems possible that a proportion of the cells, varying from one experiment to another, became multiply infected with T2r^+ phage despite the primary infection with T1, and lysis inhibition of these cells as described by Doermann (3) ensued. It was determined that about 96% of the cells were infected five minutes after the addition of T1 virus in the ratio of five virus particles per cell.

While the above experiments showed there was no stimulation of the cells by Tl, T3, or T7 viruses to break down T2r^+, it was considered possible that after infection by one of these viruses, adsorption of T2r^+ phage to the infected

TABLE I

THE BREAKDOWN OF P^{32} LABELLED T2r^+ BACTERIOPHAGE ADSORBED TO *E. coli* PREVIOUSLY INFECTED BY OTHER COLIPHAGES OF THE T GROUP

Primary infection		Interval between primary and secondary infection, min.	Infecting ratio of labelled T2r^+ phage to cells	% breakdown of T2r^+ phage
Phage	Infecting ratio of phage to cells			
T1	10	8	3	7.0*
T2r^+	5	5	1	45††
T2r	10	5	1	47**
T3	10	7	1	4*
T4r^+	5	5	1	53††
T4r	5	5	1	52**
T5	5	33	5	46†
	10	15	1	41†
T6r^+	5	5	1	49††
T6r	5	7	1	52**
T7	10	7	1	2.1*

* *Twenty minutes after addition of labelled T2r^+ phage; lysis complete in all except T1 experiment.*

** *Thirty minutes after labelled T2r^+; lysis complete in each case.*

† *Forty minutes after labelled T2r^+; neither culture fully lysed*

†† *Sixty minutes after labelled T2r^+.*

cells might stimulate them to break down radioactive T2r^+ added shortly afterwards. The following is an example of a number of experiments designed to test this point.

A cell culture was infected by the addition of 10 particles of T1 phage per cell and five minutes later T2r^+ phage was added in the ratio of five particles per cell. After a further three minutes to allow adsorption of this phage, radioactive T2r^+ was added in the ratio of one particle per cell. Aliquots were then withdrawn from the culture at intervals and the percentage breakdown of radioactive phage determined. The results from this experiment are shown in Curve 3, Fig. 1, in which the time axis starts with the addition of labelled phage. A colony count of surviving cells five minutes after addition of T1 phage indicated that 95.5% had been infected with T1. Two minutes after the addition of radioactive T2r^+ phage the overall adsorption of T2r^+ was 97.1%. At this time a titration of infective centers on B/1 cells, a mutant strain of *E. coli* resistant to T1, demonstrated that 15% of the cells were infected with T2r^+. The culture had cleared markedly 10 min. after the addition of radioactive phage.

A repeat experiment with T1 as the primary infecting phage gave results illustrated in Curve 1, Fig. 1. In this experiment there was little or no visible lysis of the culture 32 min. after the addition of labelled T2r^+ phage. The

extended lysis period and increased breakdown indicate that, previous to the addition of labelled phage, T2r^+ infection was established in a much larger proportion of the cells here than in the first experiment.

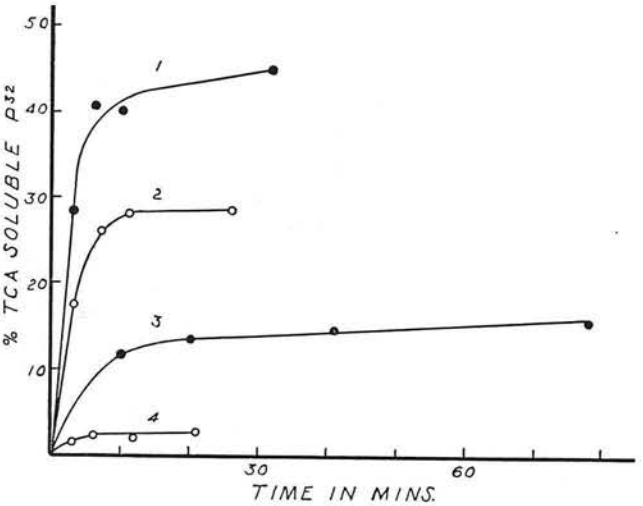

FIG. 1. The breakdown of P^{32} labelled T2r^+ bacteriophage added to previously infected *E. coli*. Curves 1 and 3, primary infection with 10 T1 phage per cell, 5 T2r^+ per cell added after five minutes followed by one radioactive T2r^+ phage per cell after a further interval of three minutes. Curves 2 and 4 obtained under identical conditions except that 10 T3 and 10 T7 particles per cell respectively substituted for primary infection with T1. Time axis starts with the addition of labelled T2r^+ phage. Lysis complete when last samples taken for Curves 2 and 4. No visible lysis of culture at last point on Curve 1, but marked lysis at 10 min. for Curve 4.

Further experiments of the same type were performed in which T3 and T7 phage were substituted for T1 in the primary infection. Curves 2 and 4 show the results obtained in two experiments with T3 and T7 phages respectively. It is interesting to note that for the experiment represented by Curve 2 the lysed culture contained 5.2×10^9 T3 phage per ml. and 1.3×10^9 T2r^+ phage per ml., while the experiment represented by Curve 4 yielded a lysate containing 9.6×10^9 T7 phage per ml. and less than 10^7 T2r^+ phage per ml. Similar results were obtained in other such experiments.

Discussion

The results presented in this paper demonstrate that the stimulation breakdown of labelled T2r^+ phage induced by previous infection of the cells with T2r^+ is also brought about by previous infection with T4r^+, T6r^+, T2r, T4r, T6r, and T5 coliphages. These phages are no less efficient that T2r^+ phage in producing the effect since labelled T2r^+ adsorbed to cells infected by them

is degraded as extensively as when adsorbed to T2r^+ infected cells. Infection with T1, T3, or T7 viruses does not stimulate the cells to break down T2r^+ phage.

When T7 phage, then T2r^+ phage, and finally labelled T2r^+ phage were added to cells, with an interval of several minutes between each addition, the radioactive phage was broken down by only 3%. Apparently the adsorption of T2r^+ to these infected cells did not stimulate the cells to break down the labelled T2r^+. However, the adsorption of T2r^+ phage to cells previously infected with T1 or T3 viruses did result in some breakdown of labelled T2r^+ added shortly afterwards. The following explanation is suggested.

When T2r^+ phage was added to the cells previously infected with T1 or T3 phages it interfered with the growth of these viruses in the manner described by Delbrück and Luria (2). A considerable, and in the case of T1 a variable proportion of the cells became infected with T2r^+ virus, thus becoming stimulated. Consequently, the labelled T2r^+ phage added shortly afterwards which became attached to these cells was extensively degraded. However, T2r^+ phage adsorbed to the surfaces of those infected cells which were sufficiently advanced in their growth cycle to give T1, T3, or T7 progeny on lysis, and thus to resist the interfering effect of T2r^+, did not stimulate the breakdown mechanism. It appears probable therefore, that mere adsorption of T2r^+ phage to the cell surface may not be sufficient to induce the stimulation breakdown effect; the cell must be infected with T2r^+ virus before the effect becomes apparent.

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The Contribution of Phosphorus from P^{32} Labelled T_2r^+ Bacteriophage to its Progeny[1,2]

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It has been observed by Lesley et al. (1951) that following infection of E. coli B with P^{32} labelled T_2r^+ bacteriophage a considerable amount of isotope may be rapidly converted to a form soluble in trichloracetic acid (TCA). After single infection of cells about 5 per cent and after multiple infection 20 to 30 per cent of the P^{32} were thus transformed. Under conditions of superinfection, that is when labelled T_2r^+ phage was adsorbed to cells infected a few minutes previously with unlabelled T_2r^+, about 50 per cent of the P^{32} became TCA soluble. TCA soluble phosphorus resulted from the breakdown of phage after adsorption to the cells and since it was mostly present in the medium, did not contribute appreciably to the formation of new virus. However, a large proportion of the isotope adhered firmly to the cells and was thus available for incorporation into virus progeny. With P^{32} labelled T_6r^+ phage Putnam and Kozloff (1950) have found that about 30 per cent of the label is transferred to the progeny in multiple infection.

[1]Paper IV in a series entitled Studies on the Relationship between Virus and Host Cell.

[2]Aided by a grant from the National Cancer Institute of Canada.

The present study was undertaken to determine the contribution of phosphorus made by labelled T_2r^+ phage to progeny under conditions of single, multiple and super infection utilizing techniques to prevent readsorption of progeny phage.

METHODS

The materials and general technique have already been fully described by Lesley et al. (1950b, 1951).

To determine the contribution of parental phage phosphorus to its progeny the general method was as follows. E. coli B grown in tryptose broth was centrifuged and resuspended in 150 to 200 ml of fresh broth at 37 C at a concentration of 2×10^8 cells per ml. An accurately known quantity of phage labelled with P^{32} was added. Adsorption of phage was usually greater than 99 per cent 10 minutes after its addition. The culture was aerated at 37 C until lysis occurred, phage titrations were carried out after the lysate had been allowed to stand 18 hours at 5 C and the phage was then purified by three cycles of differential centrifugation as previously described. In several instances where the tests were applied the trichloracetic acid (TCA) soluble P^{32} in the purified phage progeny was less than 0.5 per cent and was not increased by the action of a highly active solution of crystalline desoxyribonuclease. These tests for purity will be described in more detail in a forthcoming paper. The radioactivity of the purified progeny, counts per minute (cpm) per phage particle, was determined

and this factor multiplied by the number of phage particles in the lysate gave the total amount of P^{32} associated with progeny phage. This figure as a per cent of the total amount of P^{32} added to the culture was thus the proportion of phosphorus of the infecting phage that was built into its progeny.

In several experiments the method of infection was changed in that the cells were suspended in broth at a concentration of 2×10^9 per ml. Phage was added and after 3 minutes for adsorption the culture was diluted with broth at 37 C to a concentration of 2×10^8 cells per ml. The remainder of the procedure was as described above.

The prevention of phage adsorption by ammonium sulphate. In several phases of the work it was desirable to have a simple means of preventing readsorption of T2r+ phage released by lysis in mass cultures. It was observed that little or no adsorption of phage took place if the cells were suspended in 5 per cent (w/v) ammonium sulfate solution (AS) at pH 7. E. coli adapted to growth in broth containing this concentration of AS did not adsorb T2r+ when the AS was present. Extensive trials have not been carried out to determine how long purified T2r+ remains fully infective in this medium but broth cultures lysed in the presence of 5 per cent AS showed no decrease in phage titer after standing 5 days at 5 C.

The production of T2r+ phage in multiple infection. As will be seen later a very large variation in lysate titer was obtained from multiply infected T2r+ cultures. Lysate titers from 4×10^9 to 2×10^{11} phage per ml have been obtained on different occasions from cultures infected under similar con-

ditions. It was presumed that part of the variation might be accounted for by readsorption of newly released phage on still intact cells or cellular debris during the lysis period. To investigate this point the burst sizes of cells after premature lysis at various times in a multiply infected culture were compared to the burst size determined after the culture had lysed normally. The method of premature lysis was essentially the cyanide technique of Doermann (1948, 1949).

Cells suspended in broth at a concentration of 2.3×10^9 per ml were infected with 5 $T2r^+$ per cell. After 3 minutes for adsorption the culture was diluted with broth to a concentration of 2×10^8 cells per ml and aerated at 37 C. At intervals samples were treated in each of the three following ways, the remaining culture being allowed to lyse normally. (1) Potassium cyanide (0.5 M) was added to give a final concentration of 0.01 M. Forty per cent (w/v) AS was added immediately to give a final concentration of 5 per cent and prevent readsorption of phage from the ruptured cells. (2) Potassium cyanide alone was added as in method 1. (3) Potassium cyanide was added as in method 1 followed immediately by 180 $T6r^+$ phage per cell. The samples were titrated after they had lysed, those treated by method 3 being plated on B/6 cells. The results obtained from methods 1 and 2 are illustrated in figure 1, curves 1 and 2 respectively. Method 3 gave results similar to those of method 2. It is observed that the combination of cyanide and ammonium sulphate to lyse the cells and prevent readsorption of the liberated phage gave results consistently higher than either of methods

2 or 3. With this method a six fold increase in intracellular phage was found during the 160 minutes following the end of the latent period. The amount of phage in the culture which had lysed normally was about half the amount found as intracellular phage at 180 minutes indicating that a considerable amount of virus was readsorbed after lysis. This finding has been confirmed in many such experiments.

While the action of AS on infected cells has not been investigated thoroughly some general observations might be noted here. Adsorption of T_2r^+ can be completely prevented by 4 per cent AS and only about 10 per cent is adsorbed in 10 minutes in the presence of 3 per cent AS. If AS is added to a multiply infected T_2r^+ culture to 5 per cent concentration at any time after the middle of the latent period the culture lyses 40 to 60 minutes after the addition. It is probable that intracellular growth of phage is prevented by 5 per cent AS since if the salt is added before the middle of the latent period lysis does not occur; if added later than 13 minutes after multiple infection the phage yield in the resulting lysate is progressively greater the later the addition. AS lysates were not completely clear to the eye but always retained a distinctly opalescent and somewhat oily appearance.

The purification of T_2r^+ phage from AS lysed cultures required the introduction of a minor but necessary modification in the method described by Lesley et al. (1950 b). The lysate was centrifuged for 60 minutes at 22,000 g without a preliminary low speed clarification run, and the supernatant was poured off.

Enough saline, buffered to pH 7 with 0.01 M phosphate, was added to cover the pellet and the phage was allowed to elute over a period of 60 to 90 minutes. The large pellet of cellular debris was then removed with a fine tipped pipette and the remainder of the purification was as previously described. An additional small amount of phage could often be recovered by resuspending the debris pellet in saline allowing it to stand 18 hours at 5 C and removing the debris by centrifuging 15 minutes at 6,000 g. The phage remaining in the supernatant was then sedimented at 22,000 g.

RESULTS

Transfer of P^{32} to progeny in multiple infection.

In table 1 are shown results for the transfer of P^{32} from T_2r^+ and T_4r^+ viruses to their progenies with various infecting ratios. The results vary from 3.7 to 35 per cent contribution but there is no correlation with infecting ratio. It is, however, noteworthy that the lower results for parental contribution were obtained when the lysate titers were relatively low. Since the calculation depends directly on the lysate titer any loss of progeny phage by readsorption after lysis would be reflected in a lower transfer of P^{32}.

An experiment was carried out, therefore, in which multiply infected cells were prematurely lysed at different times after infection and readsorption was prevented with ammonium sulfate. A broth culture at 2.5×10^9 cells per ml was infected with labelled T_2r^+ phage at a ratio of 7 particles per cell. After 3 minutes for adsorption the culture

was diluted with warm broth to 2 x 10^8 cells per ml and aeration was continued at 37 C. Ninety-seven per cent of the phage was adsorbed in four minutes. At 21, 50 and 110 minutes after infection 100 to 150 ml aliquots of culture were run into a mixture of 40 per cent AS and 1.5 M potassium cyanide sufficient to give a final concentration of 5 per cent AS and 0.01 M potassium cyanide. The remainder of the culture was aerated until lysis occurred. The four lysates were stored at 5 C for 18 hours after which they were titrated and the phage was purified. The results are shown in figure 2. At 22, 51, 113 minutes after infection and in the final lysate the amounts of TCA soluble P^{32} in the culture were 6, 8, 24 and 53 per cent respectively of that added. While there was a decrease in the specific activity of intracellular phage by a factor of 2.6 in the interval 21 to 50 minutes after infection, the amount of P^{32} contained in this phage remained essentially constant at about 21 per cent of that added up to 110 minutes. The progeny in the final normal lysate contained 10 per cent of the P^{32} and this decrease in transfer is consistent with the greatly decreased burst size found for this lysate.

A second experiment was carried out in the same manner using labelled T_2r^+ phage at an infecting ratio of 2.2 particles per cell. Ninety-six per cent of the phage was adsorbed at 10 minutes. Aliquots of culture were removed at 22, 30, 40 and 50 minutes after infection for premature lysis

and determinations of P^{32} in the progeny phage. The results are presented in table 2. In this experiment there was a 4.4 fold decrease in the specific activity of progeny between 22 and 50 minutes after infection confirming a similar trend in the previous experiment. It is possible that there is a trend towards an increased contribution of P^{32} to phage at 40 minutes as compared to that at 22 minutes, and a similar but less pronounced trend is apparent in figure 1. However, it is felt that the variations may result from the numerous experimental errors possible in this type of experiment and that the actual transfer of P^{32} in the second experiment is essentially constant at an average value of 33 per cent over the period 22 to 40 minutes after infection. It is worth noting that the burst sizes in the second experiment are more than double those in the first over the period 22 to 50 minutes. Since readsorption of phage was prevented in both cases, the burst size in multiple infection is influenced by other factors as well as readsorption of progeny.

Transfer of P^{32} to T_2r^+ progeny from superinfecting labelled T_2r^+ phage. It has been demonstrated previously (French et al., 1951) that when labelled T_2r^+ phage was adsorbed to cells which 5 minutes previously were infected with r^+ or r even numbered phages about 50 per cent of the P^{32} was rapidly converted to TCA soluble form. This extensive breakdown occurred whether the superinfecting ratio of labelled phage was 0.2 or 60 particles per cell. It was presumed that infection of a cell with an r^+ or r phage rapidly induced the

formation of a mechanism whereby T_2r^+ particles adsorbed after a short interval were extensively degraded. It seemed possible that this might be a mechanism for the mutual exclusion between even numbered phages and if this were the case a much smaller contribution of P^{32} from the superinfecting labelled phage to progeny might be expected than was obtained in the multiple infection experiments. As was mentioned in a preliminary note (Lesley et al., 1950a) labelled T_2r^+ adsorbed 5 minutes after a primary infection of cells with unlabelled T_2r^+ did, in fact, contribute only about 1 per cent of its P^{32} to the progeny. The first experiments have been extended and the results are presented in table 3.

In experiments 1 and 2 cells grown in broth to 2 x 10^8 cells per ml were multiply infected with T_2r^+ phage. After aeration at 37 C for 5 minutes labelled T_2r^+ was added and after a further 10 minutes the culture was chilled and the cells sedimented to eliminate any unadsorbed phage. The infected cells were then resuspended in fresh broth and the culture aerated until lysis occurred. P^{32} in the progeny was determined after purification as described above. In experiments 3 and 4 cells resuspended in broth at a concentration of 2 x 10^9 per ml were multiply infected then superinfected with labelled T_2r^+ and after a further 5 minutes sedimented and resuspended at 2 x 10^8 cells per ml. For experiment 5 cells at a concentration of 2 x 10^8 per ml in broth were multiply infected and after 5 minutes labelled phage was added. The culture was aerated and at 61 minutes

was prematurely lysed with the ammonium sulfate-cyanide technique. Adsorption of all added phage was 99 per cent complete at 10 minutes in this experiment.

Transfer of P^{32} to T_2r^+ progeny in single infection. It was observed by Lesley et al. (1951) that a two step form of breakdown curve was obtained when a mass culture of cells was singly infected with labelled T_2r^+ phage. The second large step in this curve was interpreted as a multiple infection type of breakdown resulting from adsorption of progeny phage in the second cycle of infection. As a logical conclusion to this assumption it was postulated that during the single infection stage labelled T_2r^+ might transfer over 50 per cent of its P^{32} to progeny. This postulate would be amenable to direct proof if the phage could be isolated quantitatively after the first cycle of infection and its isotope content determined. Numerous attempts were made by a variety of methods to separate the first generation progeny in single infection but the following type of experiment, similar in principle to one designed by Putnam and Kozloff (1950), was the only one which gave consistent results.

Cells resuspended in broth at a concentration of 2×10^9 per ml were infected with 0.15 to 0.2 labelled T_2r^+ per cell. After 2 to 5 minutes for adsorption the culture was diluted to 2×10^8 cells per ml with broth at 37 C and aeration was commenced. At 19 to 20 minutes after infection 5 to 10 unlabelled T_2r^+ particles per cell were added. This was to infect the remaining uninfected cells in the culture and to

lysis inhibit the infected cells. Addition of a further 5 unlabelled T_2r^+ per cell was made at about 30 minutes. At 90 minutes AS and cyanide were added to lyse the whole culture, the progeny was purified and the transfer of P^{32} determined. The results for eight such experiments are presented in table 4. In experiment 4 part of the culture was prematurely lysed at 60 minutes, the reminder at 90 minutes and the P^{32} transfer determined for both lysates. In experiment 5 the superinfection at 20 and 30 minutes was carried out with 10 $T4r^+$ particles per cell. The result shown for this experiment was calculated from the total phage, T_2r^+ plus T_4r^+, appearing in the lysate and purified progeny. Calculated from estimations of T_2r^+ alone in lysate and purified progeny, the parental contribution was 34 per cent. Thus the presence of unlabelled phage in the lysate resulting from the secondary infection with T_2 or T_4 virus did not significantly influence the result in this type of experiment.

Concurrently with experiments 7 and 8, in which the infecting ratios were 0.2 labelled phage per cell, two further experiments were carried out with a primary infecting ratio of 5 labelled T_2r^+ per cell. In other respects the technique was the same for the four experiments, the latter two resulting in 45 and 46 per cent transfer of parental P^{32} to progeny. It is concluded that in T_2r^+ multiple infection, and probably also in single infection, super-infection with T_2r^+ towards the end of the latent period does not decrease the phosphorus transfer of the infecting virus.

DISCUSSION

It is concluded from the data of table 4 that, on the average, about 35 per cent of the P^{32} in parental T_2r^+ phage is transferred to its progeny in single infection. A similar finding was made by Putnam and Kozloff (1950) for T_6r^+ phage. This conclusion is also supported by a study of the intracellular breakdown of T_2r^+ in single infection (to be published). However, superinfecting labelled T_2r^+ adsorbed to cells 5 minutes after infection by unlabelled T_2r^+ contributes only about 1 per cent of its P^{32} to the progeny as shown in table 3. It is probable that this exclusion of T_2r^+ from participation in progeny growth occurs by virtue of the stimulation or superinfection breakdown mechanism previously described (Lesley et al., 1951).

The amount of parental P^{32} transferred to progeny in multiple T_2r^+ infection is presumably the resultant of processes which allow a 35 per cent transfer in single infection and practically none from superinfecting phage after 5 minutes. Two possibilities may therefore be considered. Firstly, 35 per cent of the P^{32} in the first particle that enters the cell is available for use by progeny but all other particles are excluded. In this event there should be a rapid decrease from 35 per cent parental P^{32} contribution with increase in infecting ratio. Secondly, the first particle invading the cell initiates an exclusion mechanism which starts to operate after a minute or so but does not reach its peak until 4 to 5 minutes after infection. In this event it might be supposed that any number of parent virus particles can each donate 35 per cent

of their P^{32} to the progeny provided they adsorb to the cell during the lag period before the exclusion process operates. Even particles adsorbed later may contribute part of their phosphorus to progeny if they arrive at the cell before the exclusion mechanism is fully effective. In this event the average amount of P^{32} appearing in the progeny would depend on a competition between adsorption rate of the phage and the rate at which the exclusion process goes into operation.

Examination of tables 1 and 2 and figure 2 shows that 30 to 35 per cent of parental P^{32} may appear in progeny with up to a seven fold infecting ratio and Putnam and Kozloff (1950) have reported a number of experiments with three fold infection by T_6r^+ in which the parental P^{32} contribution varied between 20 to 40 per cent. It is therefore apparent that the first supposition is incorrect and that more than one phage particle can contribute to progeny in T_2r^+ infection probably because the large majority of parent particles are adsorbed before the exclusion process is set in operation. The low results of table 1 were explained earlier as probably chiefly due to readsorption of progeny phage. The results of figure 2 and table 2 support this interpretation and emphasize the importance of preventing progeny phage loss by readsorption. In any case some variation in results might be expected in multiple infection where experimental conditions vary slightly from one experiment to another and at least two delicately balanced reactions are competing for parental phage.

With reference to figure 2 the average contribution of parental phage is about 21 per cent of P^{32} to progeny between 22 and 100 minutes after seven fold infection. Table 2 shows an average of 33 per cent transfer between 22 and 40 minutes after infection with 2.2 phage per cell. Although phage production is far from complete at the end of the latent period the P^{32} contribution of parental virus has largely taken place. Thus in lysis inhibited cultures a very large proportion of the final progeny particles must contain little or no parental P^{32}.

SUMMARY

It was found that when E. coli B was infected with P^{32} labelled T_2r^+ phage under conditions in which each cell adsorbed only one virus particle about 35 per cent of the isotope appeared in the phage progeny. Labelled T_2r^+ adsorbed 5 minutes after infection of the cells with unlabelled T_2r^+ phage contributed less than 2 per cent of its P^{32} to the progeny. In multiple infection with T_2r^+ up to 46 per cent of the parental phosphorus appeared in newly formed virus. In cell cultures multiply infected with T_2r^+ phage and therefore lysis inhibited the parental phosphorus contribution to progeny was largely complete at the end of the latent period. A large proportion of the virus particles formed after the end of the latent period contained little or none of the parental phosphorus.

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TABLE I

The contribution of phosphorus from P^{32} labelled phages T_2r^+ and T_4r^+ to their progenies in multiple infection

Phage	Infecting ratio phage per cell	Lysate titer phage per ml x 10^{-9}	Percent P^{32} transferred to progeny
T_2r^+	17	16	3.7
T_2r^+	15	4.4	3.7
T_2r^+	7	90	35
T_2r^+	5	8.6	5.2
T_4r^+	5	120	30
T_4r^+	1	98	22

TABLE 2

The contribution of P^{32} to progeny phage at intervals after infection of cells with 2.2 labelled T_2r^+ phage per cell. Premature lysis with cyanide and 5 per cent ammonium sulfate.

Minutes after infection	Burst size phage per cell	cpm per progeny phage x 10^{10}	Per cent P^{32} transferred to progeny
22	40	7.0	25
30	98	3.9	34
40	183	2.6	42
50	234	1.6	33

TABLE 3

The contribution of phosphorus to T_2r^+ progeny from superinfecting P^{32} labelled T_2r^+ phage

Experiment	Primary infection T_2r^+ per cell	Super-infection T_2r^+ per cell	Minutes between primary and superinfection	Lysate titer phage per ml x 10^{-10}	Percent P^{32} transferred to progeny
1	3	7	5	8.2	1.2
2	3	17	5	3.2	1.4
3	5	5	5	4.9	1.1
4	5	5	15	3.9	1.0
5	4.8	5.9	5	6.4	2.1

TABLE 4

The contribution of phosphorus from P^{32} labelled T_2r^+ phage to its progeny in single infection

Experiment	Lysate titer, phage per ml x 10-10	Per cent P^{32} transferred to progeny
1	11	33
2	11	62
3	10	35
4	9.5[a] 12[b]	39 34
5	8.6	36
6	3.2	23
7	6.3	28
8	7.3	32

[a]Prematurely lysed at 60 minutes
[b]Prematurely lysed at 90 minutes

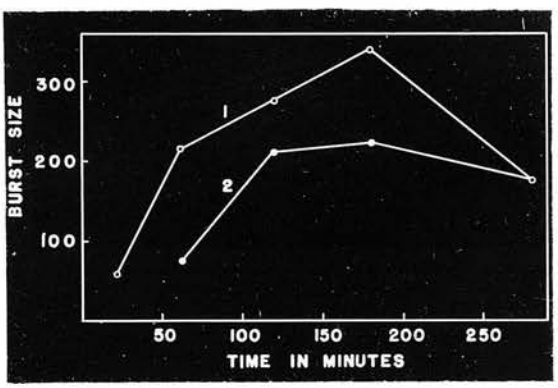

Figure 1. The burst sizes of cells prematurely lysed at intervals after multiple infection with T_2r^+ phage. Curve 1, cells lysed with 0.01 M cyanide and readsorption prevented with 5 per cent ammonium sulfate. Curve 2, cells lysed with 0.01 M cyanide. The last point on both curves gives the apparent burst size after the culture had lysed normally.

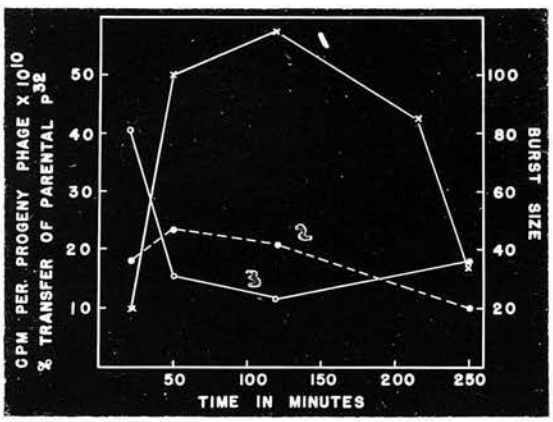

Figure 2. The appearance of parental P^{32} in the progeny at intervals after 7 fold infection with labelled T_2r^+ phage. Curve 1, burst sizes of infected cells. Curve 2, per cent of parental P^{32} appearing in the phage progeny. Curve 3, radioactivity of progeny in counts per minute per phage particle.

STUDIES ON THE RELATIONSHIP BETWEEN VIRUS AND HOST CELL V. THE BREAKDOWN OF T_2r^+ BACTERIOPHAGE[1]

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Infection of its host by P^{32} labelled T_2r^+ bacteriophage results in the rapid appearance of trichloroacetic acid (TCA) soluble P^{32} in the culture. This is formed by the degradation of the virus nucleic acid and in previous work (Lesley et al., 1951) the extent of phage breakdown under different experimental conditions was measured by the amount of TCA soluble P^{32} formed. However, only the breakdown products of relatively low molecular weight would be measured by this method, whereas the formation of fragments of all sizes from inorganic phosphate almost to intact phage particles might be expected. The present paper is mainly concerned with measurement of total labelled breakdown products present in the medium after infection with P^{32} labelled T_2r^+ virus. This quantity was determined simply by centrifuging the cells from a culture at various times after infection and estimating P^{32} in the supernatant. It is shown that under some conditions breakdown of infecting phage measured in this way is more extensive than when TCA soluble P^{32} is used as a criterion. Some observations on the intracellular breakdown of T_2r^+ in single infection are also presented.

[1]Aided by a grant from the National Cancer Institute of Canada.

METHODS

The bacteriophage used in this work was the T_2r^+ strain active on E. coli B. Materials and general methods have already been described (Lesley et al., 1950, 1951), but it should be mentioned here that two main criteria have been employed to determine the purity of radioactive phage preparations. (1) The determination of the percentage TCA soluble P^{32} in the preparation. This was usually less than 0.5 per cent as previously described. (2) The estimation of TCA soluble P^{32} after crystalline desoxyribonuclease (DNase) had been allowed to act for 60 minutes at 37 C on the purified phage preparation. Generally less than 1.5 per cent of the P^{32} was converted to a TCA soluble form by this procedure. As a control DNase was allowed to act under the same conditions on labelled phage which had been killed by heating to 70 C for 20 minutes. Up to 90 per cent of the P^{32} was thus converted to a TCA soluble form. It is interesting to note that when DNase acted on labelled T_2r^+ which had been exposed for 60 minutes to ultraviolet radiation, sufficient to reduce its infectivity to 10^{-5} per cent of the original, less than 5 per cent of its P^{32} was transformed to TCA soluble.

Unless otherwise specified the E. coli used in the following experiments was taken from a freshly grown agar slope and grown in tryptose broth at 37 C with aeration, to a concentration of 5 to 8 x 10^8 cells per ml. The culture was chilled in an ice bath, centrifuged in the cold and the cells were resuspended in fresh broth at the

required concentration. All experiments were carried out at 37 C with rapid aeration of the culture. TCA soluble P^{32} was determined as previously described. "Water soluble P^{32}", which includes all the phage breakdown products free in the medium as well as unadsorbed phage, was estimated, as follows. Samples of culture were withdrawn into chilled tubes centrifuged at 5000 g for 15 minutes in the cold to remove infected cells and P^{32} in the supernatant was measured. As with TCA soluble P^{32}, this water soluble P^{32} was calculated as a percentage of the total P^{32} added in infecting phage. As is shown later simple chilling of the samples was not sufficient to prevent further breakdown to water soluble P^{32} although this was prevented by formalin. Therefore, in most estimations of water soluble P^{32} sufficient 40 per cent formalin was added immediately after the sample was withdrawn to give a final concentration of 0.8 per cent; these samples were chilled and treated as above.

RESULTS

Breakdown of $T2r^+$ phage after single infection. When cells were infected at a ratio of one labelled phage per 10 cells the formation of TCA soluble P^{32} during the first 30 minutes was as shown in curve 3, figure 1. The formation of water soluble P^{32} is illustrated in curve 1, figure 1. Water soluble P^{32} was also determined on samples to which formalin was added immediately after withdrawal from the main culture and the results are represented

by curve 2, figure 1. In this type of experiment adsorption of phage was usually greater than 99 per cent at 10 minutes.

While broth curves 1 and 2 are consistent in showing that the initial breakdown of infecting phage amounted to about 8 per cent the second rise in curve 1, indicating release of further phosphorus from the cells, began 5 minutes before that of curve 2. This suggests that the true rise was at the end of the latent period as shown in curve 2, and the earlier increase shown in curve 1 resulted from the continuing metabolism of the infected cells during subsequent manipulation. It was concluded that the addition of formalin to the water soluble P^{32} samples effectively inhibited further breakdown.

In a previous paper it was shown that a culture infected with one T_2r^+ particle per 5 cells gave a two step breakdown curve for TCA soluble P^{32}. The second step began at 25 to 30 minutes and rose to about 23 per cent at 70 minutes. In the present work it has been found that the water soluble P^{32} curve is also two step in shape. The second increase began at 22 to 23 minutes, as shown in figure 1, and rose to a second plateau of 50 to 55 per cent at about 60 minutes. When the infecting ratio was one phage particle per 20, 50 or 100 cells similar curves for water soluble and TCA soluble P^{32} were obtained.

During the latent period of single infection breakdown did not amount to more than 10 per cent but it seemed possible that the

virus might be extensively degraded and the fragments adhere firmly to the cell. If such were the case NDase added shortly after infection might be expected to digest these fragments and thus increase the TCA soluble P^{32} fraction. The following experiment was designed to test this point. Cells resuspended in broth were infected with one labelled phage per cell. Two minutes later a highly active solution of DNase was added, the final cell concentration being 2×10^9 per ml. At intervals during the 20 minutes following infection aliquots were removed for the estimation of TCA soluble P^{32}. After 10 minutes 99.5 per cent of the phage was adsorbed. The breakdown curve was similar to curve 3, figure 1, indicating that DNase had no effect on the adsorbed phage.

That adsorbed phage can, under certain conditions, be extensively digested by DNase is demonstrated by the following experiment. A freshly grown broth culture of cells was heated in a boiling water bath for 10 minutes after which no cells were left alive. The cells were sedimented, washed once with saline and resuspended in broth. Labelled T_2r^+ in broth was added to give an infecting ratio of 2 phage per cell at a concentration of 2.4×10^9 cells per ml. After 3 minutes for adsorption the culture was diluted to 2×10^8 cells per ml with warm broth and allowed to stand at 37 C. Five minutes after its addition 99.8 per cent of the phage and 91.8 per cent of the P^{32} were adsorbed. To an aliquot of this culture DNase was added. After 35 minutes at 37 C 56 per cent of the added P^{32} had been con-

verted to TCA soluble form and 71 per cent remained in the supernatant after centrifuging out the cells. In the control culture, after 35 minutes at 37 C, there was no TCA soluble and 16 per cent water soluble P^{32}. Thus adsorption of phage to heat killed cells resulted in a modification of the virus particle which enabled it to be extensively digested by the nuclease while still adsorbed to the cell.

Intracellular breakdown of $T2r^+$ phage in single infection. As a result of the above experiments it was tentatively concluded that, in single infection of live cells the nucleic acid portion of the virus had penetrated the cell. It was then of interest to determine whether an intracellular breakdown of the invading phage occurred. To investigate this point infected cells were prematurely lysed by the method of Doermann (1948, 1949).

A culture in broth at 2×10^9 cells per ml was infected with one labelled phage per 10 cells. After 90 seconds for adsorption the culture was diluted to 2×10^8 cells per ml with broth. At intervals samples were withdrawn for estimation of TCA soluble P^{32}. Other aliquots were added to tubes containing a mixture of potassium cyanide and concentrated $T4r^+$ phage to give a final concentration of 0.01 M cyanide and 300 $T4r^+$ particles per cell. After standing 60 minutes at 37 C and 18 hours at 5 C to ensure that lysis was complete each lysate was divided into three parts; (1) was precipitated with TCA, (2) was centrifuged at 5000 g for 15 minutes,

(3) was incubated for 60 minutes with DNase at 37 C and then precipitated with TCA. Control samples incubated without DNase showed no increase in TCA soluble P^{32}. Curves 1, 2 and 3, figure 2 show the amounts of P^{32} remaining in the respective supernatants at the various times. Curve 1 also represents the TCA soluble P^{32} when samples of the culture were precipitated without further treatment.

This and other experiments showed that the TCA soluble P^{32} fraction was not increased by breaking open the cells. The P^{32} remaining in solution after TCA precipitation of a culture by the standard technique employed in these studies therefore gives an accurate measure of the amount of TCA soluble P^{32} formed in single infection. In experiments of a similar nature this has been verified for multiple infection also. Twenty-two minutes after infection 55 per cent of the P^{32} was converted to TCA soluble by DNase action indicating an extensive intracellular breakdown of infecting phage. Considerable breakdown, in fact, appears to take place within 2 to 3 minutes after infection since marked action by DNase was apparent in the earliest lysates. In another experiment of the same type 69 per cent of the P^{32} was digested by DNase in the 22 minute lysate. About 30 per cent of the P^{32} was degraded by DNase in the 2.5, 5 and 10 minute lysates and 50 per cent in the 15 minute lysate. Lysis may have been incomplete in the earlier samples since only 90 minutes at 5 C were allowed for clearing of the cultures.

Since 50 to 69 per cent of the P^{32} could be digested by DNase

in the 22 minute lysates it is concluded that this amount of parental P^{32} was not contained in newly formed phage at the end of the latent period. This is confirmation of direct estimations by French et al.(1952) which indicated that T_2r^+ progeny in single infection contained about 35 per cent of parental phosphorus.

Breakdown of superinfecting T_2r^+ phage. As described previously (Lesley et al.,1951) when cells are first infected with T_2r^+ phage and after an interval of 5 minutes are superinfected with labelled T_2r^+ 50 per cent of the label rapidly becomes TCA soluble. To investigate this breakdown of superinfecting phage the following experiment was carried out. Two tubes containing cells suspended in broth at a concentration of 3×10^9 per ml received unlabelled T_2r^+ phage in the ratio of 5 particles per cell. Ninety seconds later for the first tube and 6 minutes later for the second 3 labelled T_2r^+ particles per cell were added. A third similar tube received 5 unlabelled and 3 labelled particles per cell added simultaneously as a control. Each culture was aerated at 37 C. Three minutes after addition of labelled phage the contents of each tube were diluted with warm broth to 2×10^8 cells per ml and aeration was continued at 37 C. Measurements of TCA and water soluble P^{32} were made at intervals on each culture and the results for the control and 6 minute superinfection are shown in figure 3. It is apparent from these curves that water soluble P^{32} in the control was greatly in excess of TCA soluble P^{32}. With superinfection at 90 seconds there was little change in the water soluble P^{32}

curve but the TCA soluble P^{32} curve rose to 20 per cent at 10 minutes and did not increase further. For the sake of simplicity the 90 second curves are not shown in the graph. As the interval between primary and superinfection increased the TCA curve approached more closely to the water soluble P^{32} curve and with a 6 minute interval the maximum breakdown was about 55 per cent water soluble and 40 per cent TCA soluble P^{32}. A superinfection interval of 12 minutes did not increase the breakdown in this type of experiment.

When primary infection was carried out with cells at a concentration of 2×10^8 per ml the superinfecting phage was usually broken down to the extent of 60 to 65 per cent water soluble and 50 per cent TCA soluble P^{32} with an interval of 5 minutes between infections. It is not known why the maximum superinfection breakdown is less with the more concentrated cell suspension, although inadequate air supply may be a contributing factor. It is, incidentally, of interest to note that superinfection breakdown is not decreased by the presence of 0.01 M citrate in the culture.

Previous work (French et al., 1951) showed that superinfection breakdown of T_2r^+, as measured by TCA soluble P^{32}, did not occur when the primary infection was with either T1 or T7 phages. However, it appeared possible that such breakdown might be observed if water soluble P^{32} was used as a measure. In several experiments one labelled T_2r^+ per three cells was added to a culture at 2×10^9 cells per ml infected 5 minutes previously with 5 particles per

cell of either T1 or T7. There was no increase in water soluble P^{32} over that found in a normal single infection experiment with T_2r^+ such as shown in figure 1. With the same experimental conditions the addition of DNase 2 minutes after superinfecting T_2r^+ did not increase the TCA soluble P^{32} over that in a control where no enzyme was added.

Breakdown of T_2r^+ phage in multiple infection. In curve 3, figure 4, is shown the formation of TCA soluble P^{32} when cells at a concentration of 2×10^8 per ml were multiply infected with labelled T_2r^+. Curve 1 illustrates the formation of water soluble P^{32}, while curve 2 shows the percentage TCA soluble P^{32} formed when DNase was allowed to act on the water soluble P^{32}. Curve 2 therefore represents the TCA soluble P^{32} normally formed by breakdown of the infecting phage plus that formed by DNase action on the water soluble products of breakdown. When DNase acted upon the final lysates, without removal of debris, 56 per cent of the P^{32} became TCA soluble.

Similar results were obtained from 4, 8, 16 and 32 fold infection with T_2r^+ phage, the curves of figure 4 being average curves for the four experiments. In each case the adsorption of phage was greater than 99 per cent at 11 minutes and the cultures lysed within a few minutes of each other at 150 minutes. In the above experiments formalin was not added in the estimation of water soluble P^{32} since it was desired to determine the action of DNase on the same samples. In other experiments of a similar type

formalin was added to the water soluble P^{32} samples. At 30 minutes after infection samples with and without formalin gave similar results but at 10 minutes the formalin treated samples showed 3 to 4 per cent less breakdown. Thus the 10 minute point on curve 2, figure 4 should be displaced about 4 minutes to the right along the time axis.

In the final lysates 50 to 60 per cent of the added P^{32} could be digested by DNase and since DNase had no action on fully formed phage at least this amount of P^{32} could not have been present in the phage progeny in these experiments. It is therefore concluded that in multiple infection not more than 40 to 50 per cent of parental T_2r^+ phosphorus is contributed to progeny. This is in accord with direct estimations on the progeny by Putnam and Kozloff (1950) and French et al. (1952).

To explain the TCA soluble P^{32} curves in multiple infection it was previously suggested by Lesley et al. (1951) that the first phage particles adsorbed to the cells are broken down only to about 5 per cent but that this infection initiates the superinfection breakdown mechanism. Phage adsorbed to the cells after two or three minutes are then broken down by about 50 per cent, the curve being the average breakdown of all the phage particles. It might then be expected that if phage were very rapidly adsorbed to the cells in multiple infection most of the particles would escape the extensive breakdown and TCA soluble P^{32} would be decreased to the level observed in single infection. As a test of this point cells

were suspended in broth at a concentration of 2.2×10^9 per ml. Sixteen labelled T_2r^+ per cell was added and after 2 minutes for adsorption warm broth was added to give a final concentration of 2×10^8 cells per ml. Immediately after this dilution 99.5 per cent of the phage was adsorbed. Water soluble P^{32}, TCA soluble P^{32} and TCA soluble P^{32} after the action of DNase on the water soluble P^{32} fraction were determined. These results are illustrated in figure 5, curves 1, 3 and 2 respectively.

It is observed that by this technique the TCA soluble P^{32} was, in fact, depressed to 5 per cent or less during the first 20 to 30 minutes and this observation has been confirmed in many such experiments. However, the total breakdown, as represented by water soluble P^{32}, was not at all depressed. Evidence that this fraction did represent breakdown products is provided by the finding that a large portion of it can be digested by DNase. Further, titration for free phage in the culture at 5, 15, 30 and 60 minutes after infection gave results of 6.9×10^6, 3.5×10^7, 3.6×10^8 and 1.2×10^9 phage per ml respectively. Thus in the first 15 minutes at least after infection free phage made a negligble contribution to water soluble P^{32}.

In the above type of experiment the lysis period was generally prolonged and complete clearing of the culture did not occur often until 5 to 6 hours after infection. The extended lysis period was usually associated with a rapid and large increase of TCA soluble P^{32} as shown in curve 3, figure 5, and with low lysate titers, often

less than 10^{10} phage per ml. In a previous paper Lesley et al. (1951) suggested that this increase in TCA soluble P^{32} resulted from readsorption and superinfection breakdown of phage progeny on unlysed cells. While undoubtedly a considerable amount of new phage may be lost in this manner it contains only 35 per cent of the parental P^{32} and this is insufficient to account for an increase in TCA soluble P^{32} to 40 to 50 per cent during lysis. While we have not further investigated the problem it seems possible that a DNase is liberated into the medium during the long lysis period and transforms the water soluble P^{32} fraction into TCA soluble.

In another set of experiments broth grown cells were washed twice with 0.1 M sodium chloride containing 10^{-3} M phosphate buffer (pH 7.0) and resuspended in the same medium at a concentration of 2×10^9 cells per ml. Labelled phage was added at a ratio of 4 particles per cell and after 5 minutes for adsorption, the mixture was diluted with broth to give a final concentration of 2×10^8 cells per ml. The breakdown curves for TCA and water soluble P^{32} (formalin added) were similar to those shown in figure 5.

DISCUSSION

The hypothesis that superinfection breakdown of $T2r^+$ phage occurs in at least two steps is supported by the following argument. Increase of adsorption rate in multiple infection by the use of higher cell concentrations reduced TCA soluble P^{32} formation almost to the level encountered in single infection. However, total breakdown, as measured by water soluble P^{32}, remained approximately the same regardless of the rate at which the phage was adsorbed. Similar conclusions may be drawn from the superinfection type of experiment. When the interval between infection and superinfection was short water soluble P^{32} was greatly in excess of TCA soluble P^{32}. As the interval was increased the two curves approached more closely together until with a 4 minute interval total breakdown P^{32} was only about 15 per cent greater than TCA soluble P^{32} after the curves had attained their maximum values.

It seems then that infection of the cell by $T2r^+$ may first stimulate the very rapid formation of a mechanism which breaks down superinfecting $T2r^+$ to large fragments. If the interval between infection and superinfection is short enough, such as with multiple infection carried out in a concentrated cell suspension, these fragments are released more or less rapidly into the medium as TCA insoluble P^{32}. As the interval before superinfection becomes longer a second breakdown mechanism is initiated which digests an increasing proportion of the initial breakdown products to TCA

soluble materials before they are released into the medium. When both breakdown reactions have reached their maximum efficiency about 4 minutes after infection most of the superinfection breakdown products appear as TCA soluble P^{32}.

In view of Dulbecco's findings (1952) on the exclusion of T_2r phage by T_2r^+ it seems probable that superinfection breakdown provides the mechanism for mutual exclusion between the even numbered coliphages. However, it is not the means whereby T_2 is excluded by T1 or T7 infected cells. This was indicated by our previous observations (French et al., 1951) that superinfection breakdown of T_2r^+, as measured by TCA soluble P^{32}, was not induced by T1 or T7. The present work confirms this view since total breakdown P^{32} under the same conditions was less than 10 per cent. It will be of interest to determine whether the exclusion of T1 and T7 phages from T2 infected cells could be the result of a breakdown mechanism.

In considering the method whereby T_2r^+ phage invades the cell it seems fair to assume that the same forces which bind phage to heat killed bacteria operate also in its adsorption to viable cells. Under the conditions used here attachment of phage to heat killed cells has apparently progressed beyond the first step of electrostatic binding described by Garen and Puck (1951), and the particle has been distorted in such a way that the nucleic acid moiety becomes susceptible to attack by DNase. If

the same process is involved in infection of live cells the nucleic acid of the virus must be rapidly utilized in some way that renders it inaccessible to DNase action. Although about 8 per cent of the phosphorus is lost in breakdown products, three pieces of evidence together suggest that the remainder of the phage nucleic acid penetrates the cell in single infection. (1) DNase added to a culture 2 minutes after infection releases no TCA soluble P^{32}. (2) After premature lysis of cells at least 50 per cent of the P^{32} may be digested by DNase as early as 2.5 minutes after infection suggesting an extensive intracellular breakdown. (3) Previous work has shown (Putnam and Kozloff, 1950; French et al., 1952) that 35 per cent of the parental P^{32} is incorporated into the progeny demonstrating that at least this amount of P^{32} must have entered the cell.

No direct evidence is yet available on the fate of phage protein after infection. However, Herriott (1951) has observed that the nucleic acid of T2 phage can be almost completely separated from a protein jacket by osmotic shock and has suggested that the properties of specific adsorption, prevention of host multiplication and lysis may be limited to phage protein or the small fraction of lipid.

As a guide to future work we visualize the following as some of the events in phage infection. The particle attaches to the cell by electrostatic bonds. Almost immediately an irreversible

step follows in which the protein envelope of the phage is ruptured and, if the cell has been heat killed, the phage nucleic acid can easily be attacked by DNase. If the cell is viable, however, distortion of the cell membrane permits the virus nucleic acid to pour into the interior, perhaps leaving its protein jacket at the surface. Production of new phage is then initiated. In the case of infection with even numbered phages the superinfection breakdown mechanism is also induced. If further particles adsorb within a matter of seconds practically all their nucleic acid can enter the cell; if they adsorb after a minute or two only part and after 4 minutes none of their nucleic acid penetrates the cell but appears in the medium as breakdown phosphorus. In this view the genetic functions which promote infection and the associated phenomenon of mutual exclusion are properties of the virus nucleic acid. There may well be, therefore, a close analogy between phage infection and the action of the pneumococcal transforming principle (McCarty et al., 1946) as has been suggested several times, for example by Anderson (1946). One of the most interesting problems suggested by this hypothesis will be to determine the fate of the protein moiety of infecting phage.

ACKNOWLEDGMENT

We wish to express our debt to Dr. A. D. Hershey for some very helpful discussions during the course of the work.

SUMMARY

After single infection with P^{32} labelled T_2r^+ phage about 10 per cent of the label appeared in the medium as breakdown products of the virus. Breakdown of labelled T_2r^+ phage adsorbed to the cell 4 minutes after a primary infection with unlabelled T_2r^+ amounted to about 60 per cent of the P^{32}. Evidence is presented which suggests that this superinfection breakdown may occur in at least two stages. Firstly, a degradation of the virus to large P^{32} containing fragments and secondly, further breakdown of these to trichloracetic acid soluble P^{32} which is then released into the medium. While T_2r^+ adsorbed to heat killed cells may be extensively digested with desoxyribonuclease this is not the case when the phage is adsorbed to viable cells in single infection. However, when singly infected cells were prematurely lysed 2.5 minutes after adsorption of labelled phage about 50 per cent of the P^{32} was digested with desoxyribonuclease indicating an extensive intracellular breakdown of the virus nucleic acid.

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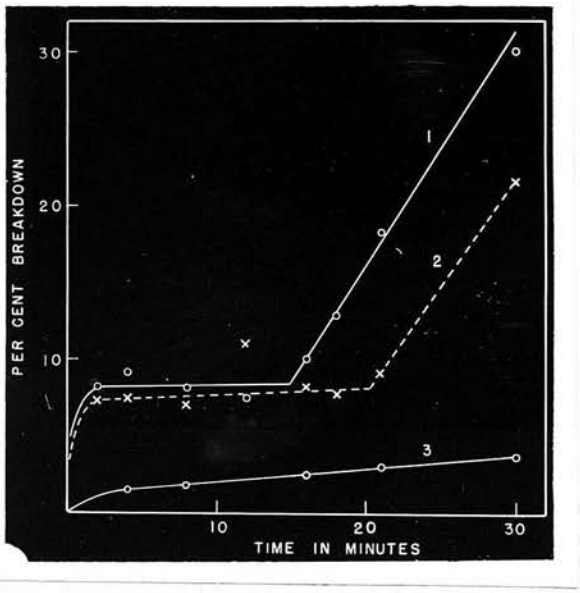

Figure 1. Breakdown of T2r+ phage in single infection. Curve 1, water soluble P^{32}, Curve 2, water soluble P^{32} after addition of 0.8 per cent formalin to the samples. Curve 3, TCA soluble P^{32}.

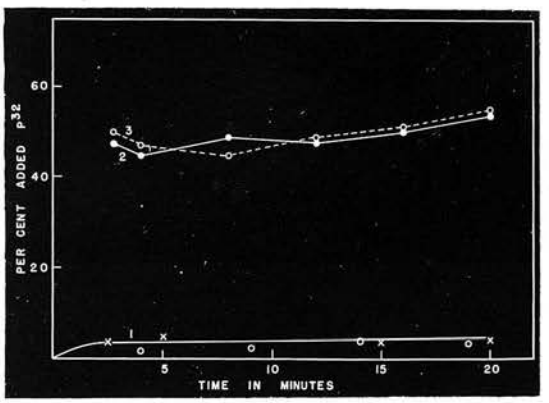

Figure 2. The release of P^{32} from cells prematurely lysed after single infection with labelled T_2r^+. Curve 1, TCA soluble P^{32} after the action of desoxyribonuclease on the lysates. Curve 2, water soluble P^{32} after removal of cellular debris by centrifuging. Curve 3, TCA soluble P^{32} in the culture before and after premature lysis.

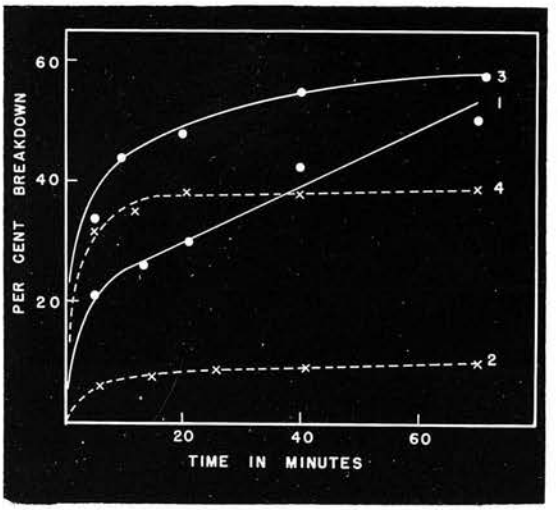

Figure 3. Breakdown of labelled T_2r^+ after superinfection of cells previously infected with unlabelled T_2r^+ phage. Curves 1 and 2, water soluble and TCA soluble P^{32} respectively in the control culture infected simultaneously with labelled and unlabelled phage. Curves 3 and 4, water soluble and TCA soluble P^{32} with a 6 minute interval between primary and superinfection.

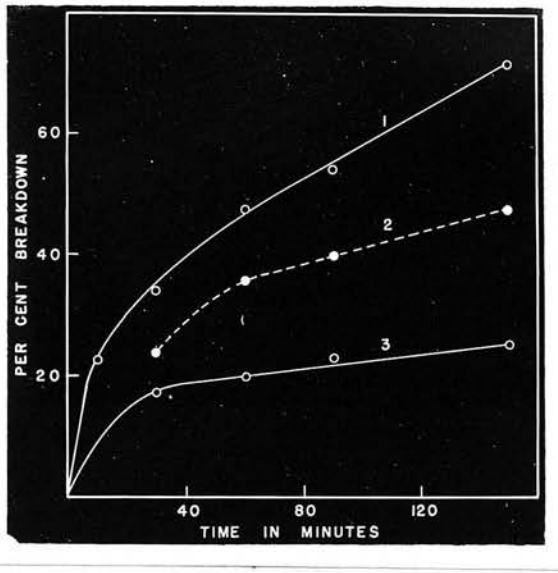

Figure 4. Breakdown of labelled T_2r^+ when cells at a concentration of 2×10^8 per ml were multiply infected. Curve 1, water soluble P^{32}. Curve 2, TCA soluble P^{32} after desoxyribonuclease acted on the water soluble P^{32} fraction. Curve 3, TCA soluble P^{32}.

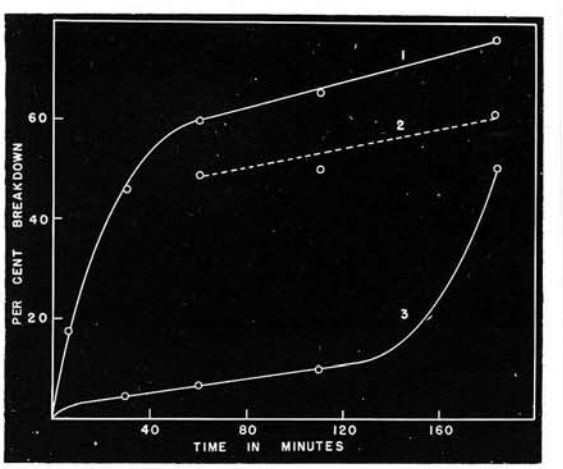

Figure 5. Breakdown of labelled T_2r^+ when cells at a concentration of 2 x 10^9 per ml were multiply infected. Curve 1, water soluble P^{32}. Curve 2, TCA soluble P^{32} after desoxyribonuclease acted on the water soluble P^{32} fraction. Curve 3, TCA soluble P^{32}.

STUDIES ON THE RELATIONSHIP BETWEEN VIRUS AND HOST CELL. VI. THE FATE OF T2 BACTERIOPHAGE INACTIVATED WITH ULTRA VIOLET RADIATION[1].

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Previous papers in this series (Lesley et al., 1950; French et al., 1951; Graham et al., 1952) have been concerned with the breakdown of $T2r^+$ bacteriophage active on E. coli B. It was found that shortly after adsorption of P^{32} labelled virus to the host the particle was broken down releasing P^{32} containing components into the medium. The greater part of this breakdown phosphorus was soluble in 5 per cent trichloracetic acid (TCA) and consequently was of relatively low molecular weight. Under conditions of single infection breakdown of the invading virus amounted to about 10 per cent. However, if the cells were first infected with unlabelled $T2r^+$ phage and 5 minutes later labelled $T2r^+$ was adsorbed the latter was rapidly broken down by about 62 per cent. This superinfection breakdown reaction has been suggested as a possible mechanism for mutual exclusion between the even numbered T phages by Delbrück and Weigle (1951). It was of interest to find whether T2 phage inactivated with ultra violet radiation would also initiate the breakdown mechanism when adsorbed to the host. Results presented here demonstrate that this is indeed the case and the findings gain added significance in the light of Dulbecco's observations (1952) that ultra violet inactivated T2 phage behaves similarly to live T2 in excluding superinfecting phage from the cell.

[1] Aided by grants from the National Cancer Institute of Canada and the W. B. Boyd Memorial Fund of the Connaught Medical Research Laboratories.

It has been found that when ultra violet inactivated labelled $T2r^+$ is adsorbed to the host simultaneously with live $T2r^+$ up to 20 per cent of the label may appear in the viral progeny. If the inactivated labelled phage was adsorbed 6 minutes after previous infection with unlabelled $T2r^+$ however, not more than 5 per cent of the P^{32} appeared in the progeny.

METHODS

All experiments were carried out in tryptose broth at 37 C. The general methods have been fully described in preceding papers of this series. As before, total breakdown products of the invading virus were measured by P^{32} remaining in the supernatant after centrifuging out the infected cells (water soluble P^{32}). The TCA soluble part of this fraction was also measured in each experiment. For irradiation a known amount of purified phage was suspended in tryptose broth and exposed in a petri dish at a distance of 20 cm from a 15 watt General Electric germicidal lamp. The dish was agitated continuously. The proportion of active to total phage after irradiation is expressed as e^{-r}, where r is the average number of hits per phage particle, in the manner described by Watson (1950). As previously mentioned by Graham et al. (1952) labelled $T2r^+$ phage irradiated to e^{-16} survival liberated about 5 per cent of its P^{32} in TCA soluble form after prolonged digestion with crystalline desoxyribonuclease (DNase).

EXPERIMENTAL RESULTS

Breakdown of inactivated $T2r^+$ in single infection. Cells at a concentration of 2×10^8 per ml were infected with labelled ultra violet inactivated phage (UVP) ($e^{-4.0}$) at a ratio of one particle for each five cells. Over the 20 minutes following infection water soluble P^{32} amounted to about 8 per cent and TCA soluble P^{32} to about 7.5 per cent of that added in the infecting phage. After 50 minutes the water soluble P^{32} was 12.4 and TCA soluble P^{32} 10.5 per cent while at 120 minutes the figures were 23.6 and

17.8 per cent for the respective fractions. Phage irradiated to e-13.5 survival gave similar results. Thus the breakdown of UVP, at least during the 20 minutes following infection, appeared to be similar to that of live T2r+ phage.

In previous work (Graham et al., 1952) it was found that DNase added to a cell culture singly infected with T2r+ phage did not increase the TCA soluble P^{32} fraction. Since phage adsorbed to heat killed cells was extensively digested by DNase it was postulated that practically the entire nucleic acid moiety of the virus rapidly penetrated into the cell. In the present work it has been found that DNase added 5 minutes after single infection with UVP (e-4.0 to e-13.5) increased the TCA soluble P^{32} to 15 to 21 per cent. Utilizing the previous argument it would therefore appear that in the case of UVP a significant amount of the phage nucleic acid remained at the cell surface in a position accessible to nuclease action.

Superinfection experiments. Figure 1 shows water soluble and TCA soluble P^{32} curves for an experiment in which cells at a concentration of 2 x 10^8 per ml were first infected with 5 T2r+ per cell and 10 minutes later one UVP (e-9.75) per cell was added. The breakdown of superinfecting UVP was greatly increased by the preliminary infection but was quantitatively somewhat less than the 62 and 50 per cent found for water and TCA soluble fractions in former experiments with superinfecting live T2r+.

It was then of interest to determine whether a preliminary infection with UVP would initiate the superinfection breakdown reaction for live labelled phage added at a later time. An experiment was therefore carried out in which cells at a concentration of 2 x 10^8 per ml were infected with 5 UVP (e-7.5) per cell and 10 minutes later one live labelled T2r+ was added. The water and TCA soluble P^{32} curves were similar in shape to those of figure 1 but the maximum heights were 48 and 36 per cent respectively.

Thus UVP is also capable of inducing the superinfection breakdown reaction.

Further experiments demonstrated that superinfecting labelled UVP adsorbed to the host a short interval after adsorption of unlabelled UVP was also extensively degraded. For example cells were infected with 5 UVP ($e^{-7.5}$) per cell and after an interval of 10 minutes one labelled UVP ($e^{-9.8}$) per cell was added. The breakdown curves were similar to those shown in figure 1, water soluble P^{32} rising to about 45 per cent and TCA soluble P^{32} to 30 per cent.

Multiple infection. As in multiple infection with live T2r+ phage (Graham et al., 1952) multiple infection with UVP gave breakdown curves intermediate between those obtained in single and superinfection experiments. Thus with 3 fold infection of cells with UVP the water soluble P^{32} rose to 20 per cent at 10 minutes and 38 per cent 80 minutes after infection. TCA soluble P^{32} was 6 and 13 per cent at the respective times. Similar breakdown curves were obtained with labelled phage irradiated to $e^{-9.8}$ and $e^{-14.1}$ survival.

The Contribution of P^{32} to $T2r^+$ progeny from superinfecting UVP.

Since UVP adsorbed to cells infected some minutes previously with live $T2r^+$ phage underwent extensive breakdown two sets of experiments were carried out to determine whether P^{32} from superinfecting UVP might appear in the $T2r^+$ progeny. A broth culture containing 2×10^8 cells per ml was divided into three portions. To the first portion one unlabelled live $T2r^+$ and 3.9 labelled UVP per cell were added together. The second portion was infected with one $T2r^+$ particle per cell and after 6 minutes 3.9 UVP per cell was added. The third portion was treated in the same manner as the second except that the interval was 11 minutes between live phage and UVP. In each case the UVP was irradiated to $e^{-9.8}$ survival.

Each culture was aerated at 37C for 70 minutes after the preliminary infection and then added to sufficient 40 per cent (w/v) ammonium sulfate and 1.7 M potassium cyanide to give final concentrations of 5 per cent ammonium sulfate and 0.01 M cyanide. As previously described by French et al. (1952) the cyanide caused the infected cells to lyse and readsorption of progeny was prevented by ammonium sulfate. The phage in each lysate was purified and the per cent contribution of isotope to progeny from UVP was determined as described elsewhere (French, et al. 1952). The results are presented in table 1, experiment 1. A second set of three experiments was carried out in a similar manner the infecting ratios being 5 live $T2r^+$ and 3.5 UVP ($e^{-2.5}$), experiment 2. In an additional experiment 6.1 $T2r^+$ and 2 UVP per cell were added simultaneously, experiment 3.

It is apparent that when UVP and live $T2r^+$ adsorbed to the cells simultaneously up to 20 per cent of UVP phosphorus appeared in the progeny. In experiment 1 the ratio of live $T2r^+$ was insufficient to infect all the cells of the culture and thus a portion of the UVP would adsorb to uninfected cells and be lost as far as its contribution to progeny was concerned. This may account for the low result of 9 percent at zero time. Both experiments 1 and 2 show that superinfecting UVP after 6 minutes contributes little P^{32} to the progeny. The results at 6 and 11 minutes are perhaps due to unadsorbed UVP or radioactive impurity carried through the purification process but in any case act as adequate controls to emphasize the significance of the transfer at zero time.

DISCUSSION

The results presented here indicate that the breakdown of UVP in single and multiple infection of the host is essentially similar to that of live T2r$^+$ phage (Graham et al, 1952). UVP can also induce the extensive breakdown of superinfecting live or irradiated T2 phage. The UVP was irradiated sufficiently to eliminate the possibility that a significant number of cells became infected as a result of multiplicity reactivation. It therefore appears that the infectious property of the invading particle may be removed by irradiation with little effect on its ability to induce the stimulation breakdown reaction in the host cells. Dulbecco (J. Bact., in press) has recently found that T2 phage heavily irradiated with ultra violet light is nearly as efficient as active phage in preventing growth of superinfecting live T2. It is therefore difficult to avoid the conclusion that mutual exclusion among the even numbered phages is the result of breakdown of the superinfecting phage. As further support for this hypothesis Doermann (unpublished results) has found that the exclusion of T2r from r$^+$ infected cells parellels the increasing breakdown of T2r as the superinfecting interval is increased.

When cells were infected simultaneously with UVP and live T2r+ up to 20% of the UVP phosphorus appeared in the T2r$^+$ progeny. This confirms a similar finding by Kozloff (1952) with N^{15} labelled T6 phage. Under these conditions there is no mutual exclusion by UVP as Dulbecco (loc. cit.) has found. It therefore seems possible from these results that at least part of the phosphorus contribution from parent to progeny is non-specific in nature. Support is thereby lent to the contention of Maaløe and Watson (1951) that no specific part of the parental phosphorus is necessary for the

growth of new virus.

The exclusion of phosphorus of superinfecting UVP after 5 minutes from participation in progeny formation is similar to the result obtained with live superinfecting T2r$^+$ (French et al, 1952). It seems possible, therefore, that transfer of phosphorus to the new virus may be prevented by the superinfecting breakdown mechanism.

SUMMARY

T2r$^+$ phage heavily irradiated with ultra violet radiation is almost as efficient as active T2r$^+$ in inducing the stimulation reaction in the host for the breakdown of superinfecting T2r$^+$ phage.

When ultra violet irradiated and active T2r$^+$ phage are adsorbed to the host together up to 20 per cent of the phosphorus of the killed phage may appear in the progeny. With an interval of six minutes between preliminary infection with live T2 and secondary adsorption of irradiated phage, about 5 per cent of the phosphorus in the latter is contributed to the progeny.

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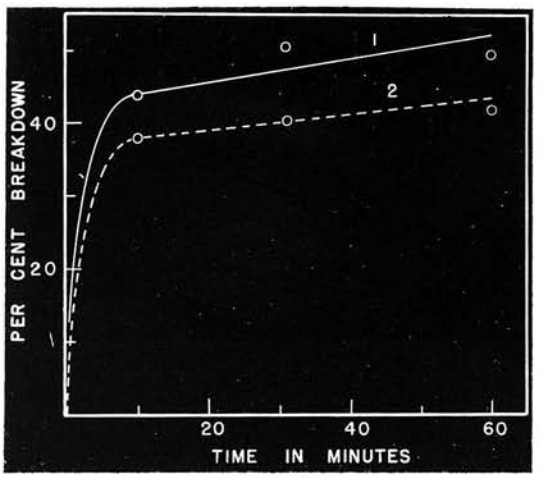

Fig. 1 The breakdown of UVP adsorbed 5 minutes after infection of the cells with live T2r$^+$ phage. Curve 1, water soluble P^{32}. Curve 2, TCA soluble P^{32}.

TABLE 1

Contribution of P^{32} to T2r+ progeny from UVP added at intervals after infection of cells with T2r+.

Minutes between infection with T2r+ and superinfection with UVP	Per cent of P^{32} from UVP in T2r+ progeny		
	Experiment 1	Experiment 2	Experiment 3
0	9.0	22	19
6	5.2	3.2	---
11	3.7	4.3	---

Reprinted from THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, St. Louis.
Vol. 36, No. 1, Pages 146-147, July, 1950. (Printed in the U. S. A.)

AN APPARATUS FOR PIPETTING RADIOACTIVE SOLUTIONS

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FIGURE 1 illustrates a device which has been found useful for the accurate and safe pipetting of tracer amounts of radioactive solutions in our studies on the uptake of radioactive phosphorus by influenza virus.[1, 2]

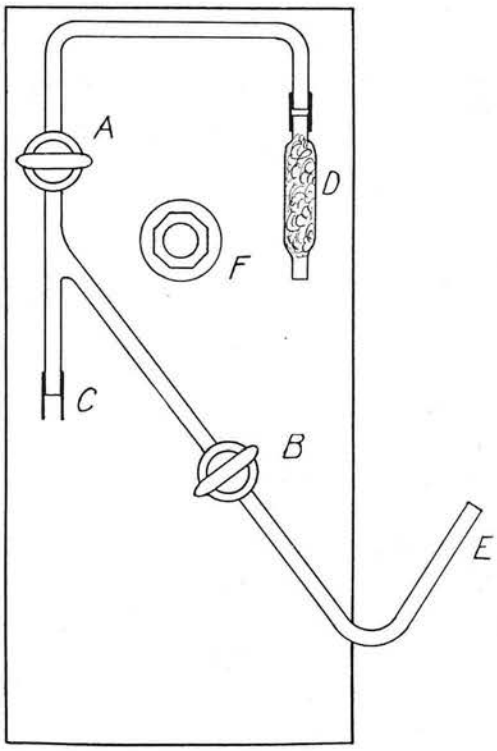

Fig. 1.—Apparatus for pipetting radioactive solutions.

The apparatus is constructed of 1 mm. bore capillary tubing with capillary stopcocks at positions *A* and *B* and is clamped securely to a piece of three-ply wood, 6 in. by 18 in., with several steel spring clips. Holes about 1½ in. in diameter are drilled in the board to accommodate the stopcocks. The upturned end, *E*, is bent back about 40 degrees from the plane of the wooden support. A small tube, *D*, packed with cotton wool, is joined to the capillary with rubber tubing. The threaded end of a short length of 3/8 in. diameter steel rod is fixed at *F* with a bolt and washer on each side of the support; this permits the apparatus to be clamped in a vertical position to a burette stand.

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Aided by a grant from the National Cancer Institute of Canada.
Received for publication, March 20, 1950.

For use, a pipette is inserted into the short length of gum rubber tube at *C* until it makes contact with the end of the capillary tube. It is then clamped to the support with a single spring clip. With stopcock *A* open, *B* closed, solution is drawn into the pipette by applying suction through a rubber tube fastened to the open end of *D* either by mouth or with a hypodermic syringe. Stopcock *A* is then closed, and, with the index finger of the right hand closing off the open end at *E*, stopcock *B* is opened. The flow of liquid from the pipette is then controlled in the usual way by pressure of the finger at *E*.

It has been found in practice that the accuracy and control over liquid flow are equivalent to those in normal pipetting technique. Since it is not essentially an apparatus for protection from radiation, this device should be used only for pipetting tracer amounts of radioactive solutions.

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SUMMARY

The work on influenza virus demonstrated that it was possible to label this virus growing in embryonated eggs when P^{32}, as inorganic phosphate, was placed in the allantoic fluid. Control experiments showed that the P^{32} was incorporated into the structure of the virus during its growth. Chemical analysis supported this conclusion and further showed that the P^{32} label was distributed between phospholipid and nucleic acid the latter having four times the specific radioactivity of the former.

It was found that the specific radioactivity of influenza virus rose in a linear fashion with the amount of P^{32} placed in the allantoic fluid. In the early work there was a limit to the amount of radioactivity that could be injected into each embryonated egg because of the toxicity of the isotope. This placed a practical limitation on the amount of isotope that could be incorporated into the virus and hence limited the usefulness of the labelled virus as an experimental tool. It was later found that the toxicity of the P^{32} for embryonated eggs was not due to radiation damage, as was at first thought, but perhaps to an unknown toxic factor present in early shipments of P^{32} from Chalk River. As is shown, this toxic material was not present in later shipments of P^{32} and it should now be possible to obtain labelled influenza virus with up to 1000 times the specific activity of the earlier preparations. This has, in fact, recently been accomplished by Blank, Henle and

their co-workers at Philadelphia (private communication from Dr. Blank) utilizing the methods developed by Graham and McClelland.

While the investigation into influenza virus growth, which was originally planned, has not yet been carried out, the work did demonstrate for the first time that it was possible to incorporate an isotope into an animal virus. It is probable that the methods described may be extended to other viruses. Further, any projected study of influenza growth in the embryonated egg requires that information be available on the chemical composition of the host cell. The present work provides some chemical analyses of the phosphorus constituents of the allantoic membrane and some data on the uptake of P^{32} by the normal and infected membrane.

The bacteriophage work presented here describes the methods used to obtain purified T2 coliphage labelled with P^{32}, and provides evidence that the label was incorporated almost exclusively in the desoxyribonucleic acid moiety of the virus. Virus preparations were obtained with high enough specific radioactivity to enable them to be used in further studies. These studies were concerned with the fate of the desoxyribonucleic acid (DNA) of the virus particle after infection of the host cell.

Essentially the results showed that when the cell was infected with labelled T2 phage about 10% of the P^{32} appeared in the medium within 5 minutes as breakdown products

of phage DNA. The breakdown products were composed of two fractions one soluble in 5% trichloroacetic acid (TCA) and one insoluble in this reagent. Thus the breakdown of the invading phage particle resulted in liberation of a mixture of phosphorus compounds varying in complexity from inorganic phosphorus to macromolecules almost as large as the phage particle. There was in addition an extensive intracellular breakdown of the invading particle.

When the labelled T2 phage was adsorbed to a cell which had been infected 5 minutes previously with an unlabelled T2 particle the superinfecting particle was broken down to the extent of about 60%. This effect was found with the related phages T2r$^+$, T4r$^+$, T6r$^+$, their mutants T2r, T4r and T6r and the unrelated phage T5. It was also found that T2 phage inactivated with ultra-violet radiation adsorbed to the host and induced the superinfection breakdown reaction; superinfecting labelled T2 phage was broken down whether it was live or killed with ultra-violet radiation. T1, T3 or T7 phages however did not stimulate the cells to breakdown superinfecting T2 virus.

About 35% of the phosphorus of the infecting T2 phage particle appeared in its progeny upon lysis of the cell. However, if the labelled T2 particle was adsorbed 5 minutes after a preliminary infection with unlabelled T2 less than 2% of the isotope appeared in the progeny. When P^{32} labelled ultra-violet killed T2 phage was adsorbed to the cell at the same time as unlabelled live T2 phage, the

cells became infected and up to 22% of the P^{32} appeared in the progeny. If there was an interval of 5 minutes between infection with live T2 and superinfection with ultra-violet killed T2, 5% or less of the phosphorus of the latter appeared in the progeny.

When cells were multiply infected with labelled $T2r^{+}$ phage, resulting in the condition of lysis inhibition, about 35% of the label was contributed to the progeny and this transfer was essentially complete in 22 minutes. However, four to six times the amount of phage was formed after 22 minutes as before and it is therefore probable that this extra phage contained none of the phosphorus of the parent.

While experimental evidence is not presented, the methods described here have been extended to T7 phage. The breakdown picture for this virus is quite different than for T2. In single infection with labelled T7 the breakdown of the particle amounts to about 10% as measured by extracellular breakdown products. Superinfection with labelled T7 5 minutes after a preliminary infection with unlabelled T7 does not lead to an increased breakdown of the labelled virus. Nor does preliminary infection with T2 result in increased breakdown of superinfecting T7 phage. The superinfection breakdown phenomenon would thus appear to be restricted to T5 and the even numbered phages. It was also found that up to 20% of the phosphorus of infecting T7 phage was contributed to

its progeny.

DISCUSSION

Much of present knowledge on the mechanism of virus reproduction is the result of work on the T group of E. coli bacteriophages. Two main experimental approaches have been utilized in recent years, the genetic and the biochemical, but in spite of intensive work on the subject it is much too early to form more than a very general picture of bacteriophage reproduction. The field is yet in the phase that a hypothesis must serve largely as a guide to work in the immediate future and stands an excellent chance of being replaced by a quite different one within six months. Fortunately, however, those results which at present appear to be most significant have been amply confirmed, often in several different laboratories. Although there may be considerable difference of opinion among different workers on interpretation, there is thus little dispute about the main experimental facts. It is too early also to determine how far the arguments based on work with the coliphages will extend to the more complex animal viruses or even to other bacteriophage systems. As will be shown later, there are in fact some similarities between the various virus systems but the following discussion is limited to the behavior of the T group of E. coli bacteriophages.

Three main steps may be recognized in bacteriophage infection.

(1) Adsorption or attachment of the virus particle to the cell. This seems to be achieved primarily by the

formation of an electrostatic bond between virus and host. Under some conditions this may be reversed and the virus particle eluted from the cell. (Garen and Puck, J. Exptl. Med. 94, 177, 1951). Normally, however, the electrostatic attraction is rapidly followed by a reaction, claimed by Garen and Puck to be enzymatic, in which the virus is irreversibly held to the cell and cannot be recovered by any known means.

(2) The "latent period" during which growth of new virus is initiated inside the cell, a rapid and extensive rearrangement of the chromatinic material of the cell occurs (Murray, Gillen and Heagy, J. Bact. 59, 603, 1950; Luria and Human, J. Bact. 59, 551, 1950), cell division and growth of adaptive enzymes ~~is~~ are prevented (Cohen, Bact. Rev. 13, 1, 1949; Monod and Wollman, Ann. Inst. Pasteur, 73, 937, 1947) the mutual exclusion, or interference effect, is induced and finally mature virus particles appear in the cell. This period varies in length from 13 to 40 minutes with the different T coliphages.

(3) Lysis of the infected cell with release of the progeny of the infecting particle. On the average 150 to 300 new phage particles per cell are released, the burst size being characteristic of the particular phage used in the infection. Nothing is known with any certainty about the mechanism of the lysis phenomenon.

The present research is concerned mainly with the disappearance of the infecting phage particle, that is with events occurring at the beginning of the latent period. However, before attempting to assess the significance of this work some of the main findings of other workers will be discussed.

It has been found by Cohen (J. Biol.Chem. 174, 281, 1948) that immediately on infection of the host with T2 phage the normal process of cell division ceases and there is no further synthesis of ribonucleic acid (RNA). Protein synthesis, however, continues at a constant rate and after a 7 minute lag period desoxyribonucleic acid (DNA) synthesis recommences and continues at a rate about four fold greater than that in the uninfected cell. Upon lysis of the cell the amount of DNA formed after infection can be quantitatively accounted for by the amount found in the new phage particles. After infection not only is there no increase in RNA but the RNA already present appears to be inert since studies with P^{32} showed that, while the DNA fraction readily incorporated the isotope, there was little or no labelling of the RNA. It is postulated by Cohen (Bact. Rev. 15, 131, 1951) that the phosphorus normally utilized by RNA is shunted into the synthesis of DNA during infection.

During the formation of T2, T4 and T6 phages about 30% of the phosphorus incorporated into the virus is derived from cellular constituents already present before infection;

the remaining 70% comes from inorganic phosphate present in the medium in which the infected cells are suspended (Cohen, J. Biol. Chem. 174, 295, 1948; Putnam and Kozloff, Science, 108, 386, 1948). Ammonium sulphate in the medium may supply 80% of the nitrogen required by the growing virus and the remaining nitrogen is supplied by bacterial constituents, probably mainly by bacterial DNA (Kozloff, Knowlton, Putnam and Evans, J. Biol. Chem. 188, 101, 1951). Using a C^{14} label Weed and Cohen (J. Biol. Chem. 192, 693, 1951) have recently found that while the pyrimidines of host nucleic acid are utilized by growing T2 phage only the first quarter of the phage produced contained this host material. The remaining phage pyrimidine is synthesized de novo. The evidence further indicated that host DNA that does appear in the virus is not transferred intact but is first broken down to units no larger than nucleotides and then resynthesized into virus material.

Apparently, therefore, the synthesis of bacterial constituents that goes on in the normal cell is interrupted by phage infection. Since the new virus is built up from components synthesized partly before, but mostly after, infection, it is assumed that the new synthesis is performed by the cellular enzymes which have been directed into a new pathway by the infecting particle. This hypothesis has been further elaborated by Luria (Science, III, 507, 1950).

Chromatinic staining of E. coli revealed that the nuclear bodies suffered almost immediate disruption upon infection with T2 phage. This was followed by the formation of finely granular chromatin which was tentatively identified with phage material (Luria and Human, J. Bact. 59, 551, 1950). It is postulated by Luria that normal genetic control of cell function is lost in the destruction of the nuclear bodies after infection. However, a new control is supplied by the invading particle whereby the synthetic mechanisms of the cell are redirected by the genetic determinants of the virus. Thus synthesis of protoplasm ceases but the same enzyme apparatus is directed towards the synthesis of new virus particles. Genetic studies of phage infection support this view and give some insight into the mechanism of virus growth.

Thus it has been known for a number of years (Delbrück and Luria, Arch. Biochem. I, 111, 1942) that two unlike phages, for example T1 and T2, will not multiply in the same cell. If the cell is infected with one phage and a second unlike phage particle adsorbs, the second particle may not grow at all, or it may grow and completely suppress multiplication of the first phage. This mutual exclusion, or viral interference, is also well known with animal viruses (Henle, J. Immunol. 64, 203, 1950). Phages T2, T4 and T6 form a closely inter-related group, however, and a cell infected with two of them gives a yield in which progeny of both parents appears. Each of these phage types may appear in a parent form designated r^+ and a mutant form designated r.

That is certain genetic markers may appear in all three types. It was found by Delbrück and Bailey (Cold Spring Harbor Symp. Quant. Biol. II, 33, 1946) that when a cell was mixedly infected with two of the types T2, T4, and T6 which differed in the r^+ and r characters, for example $T4r^+$ and T2r, the yield contained both parental types and in addition two other types resulting from exchange of the genetic markers. Thus the yield contained $T4r^+$, T2r, T4r and $T2r^+$. This "recombination phenomenon" was further studied by Hershey (Genetics, 31, 620, 1946, Proc. Nat. Acad. Sci. 34, 89, 1948) and the results have led Luria (Science, 111, 507, 1950) to suggest that the parent particles divide up inside the cell into a number of discrete genetic units which recombine during subsequent growth to give the various genetic types in the progeny.

A somewhat different type of interaction is found in the phenomenon of "multiplicity reactivation" (Luria, Proc. Nat. Acad. Sci. 33, 253, 1947). Thus the adsorption to the host of a single T2 particle inactivated with ultra-violet radiation may prevent further cell division, produce characteristic intracellular changes (Luria and Human, J. Bact. 59, 551, 1950) and prevent further DNA synthesis by the cell (Cohen and Arbogast, J. Exptl. Med. 91, 637, 1950) but no growth of virus occurs. If two or more of the ultra-violet inactivated particles adsorb to the same cell intracellular virus growth may be initiated and a normal yield of progeny obtained. Luria presumes that a virus particle is inactivated by destruction of some of its genetic material. If two particles which have

received lethal hits in different sections are adsorbed to the same cell, the remaining undamaged portions may function together to initiate infection.

Experiments with many different viruses have shown (Bauer, Nature, 164, 767, 1949) that once infection of the cell occurs the infecting virus particle cannot be recovered by breaking open the cell. This has led Bauer and others to suggest that there must be a break-up of the infecting virus into a soluble phase immediately upon entering the cell. In the bacteriophage field Doermann (Carnegie Institution Yearbook, 47, 176, 1948) has found that after infection of E. coli with T2 phage no virus was present inside the cell until halfway through the latent period. The amount of virus then increases at an approximately linear rate until lysis at 22 minutes. Using mixed infection with different mutants of T2 phage Doermann also showed (ibid, 48, 170, 1949) that the first virus particles to appear in the cell were composed of the same proportion of parental and recombinant types as appeared in the final yield from the cell. Thus the reactions which set the pattern for recombination must be organized before the appearance of the first phage particles. A further deduction was that recombination occurs before or at the time of formation of the phage particles and these particles once formed undergo no further change.

Luria (unpublished results) has analysed the distribution of spontaneous phage mutants formed in individual cells during intracellular phage growth. The new mutants

were present in individual bacteria in clones, each clone containing all the mutant particles resulting from one mutation. From the distribution of the number of mutants per clone Luria suggested a phase of logarithmic reduplication of phage material during which mutants occur. Genetic recombination, however, would seem to occur after this logarithmic phase since in mixed infection experiments the particles of any one type were not distributed in clones but appeared very nearly at random in individual bacteria (Hershey and Rotman, Genetics, 34, 44, 1949; Doermann, Carnegie Institution Yearbook, 48, 170, 1949). If recombination occurred earlier, each recombinant would give rise to a clone in the same manner as a mutant. The evidence also argued against recombination occurring after formation of the final phage particle since the recombinants of reciprocal phage types formed in individual bacteria were not present in equal or correlated numbers. This finding thus supports the conclusion already reached by Doermann that after the new phage particles have been formed they undergo no further change.

A somewhat different approach has been utilized by Latarjet (J. Bact. 53, 149, 1947; J. Gen. Physiol. 31, 529, 1948). After infection of E. coli with T2 phage survival curves of the phage in infected bacteria were determined after irradiation with ultra-violet and x-radiation. Analysis of these curves led to the suggestion that during the first seven minutes after infection the invading particle remained unique and genetically intact. Thus no multiplication occurred during

this time although the data did not oppose the idea that the invading particle might subdivide into a number of smaller units. During this period, however, there was synthesized a large amount of ultra-violet adsorbing material which may have been necessary for the building of future virus particles. From the seventh to the ninth minute there was a rapid increase in x-ray resistance of the infected cell, multiplication then started and was complete by 13 minutes with an average of 100 to 150 units per cell. These units appeared to be smaller than the actual virus particle, but after 13 minutes they grew in size until at the end of the latent period they were similar to extracellular virus.

The genetic studies supported by the data of Latarjet have led Luria (Science, 111, 507, 1950) to postulate that the invading particle on entry into the cell divides into a number of genetic sub-units. This division is followed by a logarithmic reduplication of these genetic units and a final reorganization into complete mature phage particles.

In view of some of the above findings, there were indications that the infecting virus particle must undergo a profound modification upon attachment to the cell. The present work with P^{32} labelled T2 phage has provided direct confirmation of this. Practically the entire nucleic acid moiety of the virus seems to penetrate to the interior of the cell in single infection, although up to 10% of the phosphorus appears rapidly in the medium as breakdown products. The nucleic acid splits up inside the cell into fragments which are probably

still of considerable molecular size since they are insoluble in trichloroacetic acid and digestible by desoxyribonuclease. Essentially the same finding has been made by Maaløe and Watson (Proc. Nat. Acad. Sciences, 37, 507, 1951).

There was indirect evidence to suggest that while the virus nucleic acid penetrated the cell, the protein envelope remained adhering to the surface. This is a purely tentative explanation but would be readily proven by studying infection with phage labelled specifically in the protein moiety. If the hypothesis should prove to be correct, it may mean that adsorption to the cell and therefore host range specificity would be properties of the protein portion of the virus. The genetic properties of the virus which initiate infection and its attendant phenomena of virus growth and mutual exclusion would belong to the nucleic acid. The transformation of pneumococcal types (McCarty, Taylor and Avery, Cold Spring Harbor Symp. Quant. Biol. 11, 177, 1946), which may be induced by specific nucleic acids, and virus infection would be quite analagous processes in this view.

While there was a rapid degradation of the infecting virus particle, it was also found that about 35% of its phosphorus appeared in the new progeny. The same contribution of phosphorus was made to progeny when up to seven labelled virus particles adsorbed to each cell. This observation was made by Putnam and Kozloff (J. Biol. Chem. 182, 243, 1950) for T6 phage, by ourselves for T2 and T4 phage (Arch. Biochem. 28, 149, 1950) and confirmed by Maaløe and Watson)

(Proc. Nat. Acad. Sciences, 37, 507, 1951). It has recently been found in the present work that up to 20% of the phosphorus of infecting T7 phage may appear in its progeny and the parental contribution is not dependant on multiplicity of infection. These findings indicate that several parental particles may enter the cell and each make the same contribution of phosphorus to the resulting progeny. Two possible consequences arise from these observations:

(1) That the phosphorus contributed from infecting virus to the progeny represents a specific conserved portion of the parental nucleic acid necessary for the growth of new virus and which may carry the genetic characters of the parent.

(2) That the transferred phosphorus is a non-specific part of the parent which has been released by intracellular breakdown and utilized by the growing progeny from the "metabolic pool" since it happens to be in an easily assimilable state at the time it is required.

The weight of evidence is strongly in favour of the second alternative. Firstly, Maaløe and Watson (Proc. Nat. Acad. Sciences, 37, 507, 1951) have shown that first generation progeny containing 35% of parental phosphorus in turn donates 35% of its phosphorus to its progeny when used to infect the cell. There is thus a 35% transfer of parental phosphorus in each succeeding generation. If a part of the original phosphorus was being conserved as an essential part of the virus, the second generation progeny

should contain 100% of the phosphorus of the first.

Secondly, it has been found in the present work that labelled ultra-violet inactivated T2 phage adsorbed to the cell at the same time as live unlabelled T2 phage may contribute up to 20% of its phosphorus to the progeny. Thus, while the inactivated phage does not itself grow in the cell a portion of its phosphorus enters the cell and is utilized by the virus growing as a result of infection with live T2 phage. A similar result has been found for ultra-violet inactivated T6 phage labelled with N^{15} (Kozloff, J. Biol. Chem. 194, 83, 1952).

Thirdly, Kozloff superinfected T7 infected cells with N^{15} labelled T6 phage and found 5% of the label in the T7 progeny. In the present work similar experiments have been carried out which indicated that 6% of the phosphorus of T2 phage may be transferred to growing T1 phage and up to 15% of the phosphorus of superinfecting T7 phage may appear in T2 progeny. The results of Kozloff for transfer of material between unrelated phages are thus essentially confirmed in the present work. However, it is not yet certain to us whether these results indicate a real transfer since, for technical reasons, it is possible that the P^{32} of the superinfecting phage which appears in the unrelated progeny may be a radioactive impurity and not incorporated into the phage particle at all. This point will bear further study before it is definitely settled.

Kozloff (J. Biol. Chem. 194, 83, 95, 1952) has argued that the transfer of material to viral progeny from

ultra-violet inactivated phage and unrelated phages, and thus without a corresponding transfer of genetic factors, is evidence that in normal virus growth transfer of genetic characters from parent to progeny is independant of material transfer. The conclusion, although it may be correct, does not follow from these results. The growing virus which may multiply 200-fold must get its building material from cell or medium. The results merely say that components from inactive or unrelated phages adsorbed to the cell are assimilated by the growing phage and give no indication of the fate of the particle which initiates the infection.

Fourthly, the present results suggest that the first phage particles formed in T2 infection contain most of the contribution of parental P^{32}, and that at least three-quarters of the new virus may contain none. A similar observation has been made by Doermann (unpublished). It would be concluded from this that parental material is not necessary for the formation of much of the new phage.

Thus, at present, it seems probable that the formation of new virus does not require transfer of a specific and necessary material part of the parent to the progeny. There is, in fact, no evidence for the idea, proposed mainly by the geneticists, that the infecting particle breaks up into sub-units which act as a pattern for replication. Actually, the words "replication" and "self-duplication" in common use to describe the reproductive process have very little meaning in this connection and their continued use is liable to lead to the delusion that the issue has been clarified in some way.

Since any explanation at present is largely conjecture, it is just as easy to assume that the invading particle takes over control of cell metabolism and plays no further active part in virus formation. All new virus would be entirely the result of synthesis by the infected cell and even the genetic characters of the progeny would not be inherited directly but result from the direction of synthesis impressed upon the cell by the parent phage. Such a hypothesis is, however, based on work carried out largely with P^{32} labelled virus and may need drastic revision when experiments are carried out with phages labelled in specific positions with C^{14} and S^{35}.

A phenomenon which has long been obscure is that of viral interference or mutual exclusion. The results presented here may have some bearing on this process. Thus, while the T2 particle which initiates infection releases only about 10% of its phosphorus into the medium as breakdown products, a second T2 particle adsorbed 5 minutes later is extensively degraded and rapidly releases up to 65% of its phosphorus into the medium. Further, the second T2 particle makes no contribution of its phosphorus to the progeny. This phenomenon has been found with all the even numbered phages T2, T4 and T6, their fast lysing mutants and with the unrelated T5 phage. Further, it was found that ultra-violet killed T2 phage adsorbed to the host also stimulated the formation of the breakdown mechanism and either live or ultra-violet killed T2 adsorbed a few minutes later was extensively degraded. It is, therefore, postulated that infection of the cell with any one

of these viruses causes the rapid formation of a breakdown mechanism. A superinfecting virus particle adsorbed to the cell 5 minutes after the primary infection is then excluded from participation in progeny growth since it is broken down and rapidly liberated in the medium.

Weight is lent to this hypothesis by some recent findings of Dulbecco (in press, J. Bact.) who observed that the adsorption of either live or ultra-violet killed T2r$^+$ phage to the cell completely excluded the growth of T2r adsorbed 4 minutes later. More recently Doermann (unpublished results) has carried out experiments in which cells were infected with T2r$^+$ and at intervals thereafter P^{32} labelled T4r was added. The amount of P^{32} appearing in the progeny, breakdown of T4r and proportions of T2r$^+$ and T4r in the progeny were estimated. It was found that when the interval between the primary and secondary infection was increased, the amount of T4r and P^{32} in the progeny decreased as the amount of breakdown increased. When breakdown of the superinfecting phage was at its maximum there was no T4r and only a small, and probably insignificant, amount of P^{32} in the progeny. There would therefore appear to be a very close correlation between the exclusion of a second phage from the cell and its breakdown.

Although it has been postulated that this superinfection breakdown is a reaction occurring at the surface of the infected cell, and can perhaps be more readily visualized as such, there is little evidence to suggest that it might not equally well be the result of intracellular break-

down and release of the fragments into the medium. Nor is there evidence to enable one to decide about the nature of the breakdown reaction although it may result from the rapid formation of a nuclease system triggered by the invading particle.

The explanation that mutual exclusion among T2, T4, T6 and T5 phages may occur by virtue of a breakdown reaction which constitutes a barrier to penetration by the second particle has so far been readily accepted (Weigle and Delbrück, J. Bact. 62, 301, 1951; Dulbecco, J. Bact., in press). However, the mutual exclusion which occurs with the odd numbered phages, T1, T3, and T7 cannot be explained in this manner. Cells infected with T1, T3 or T7 did not break down superinfecting T2 or T7 particles, even when the second particles arrived at the cell late enough to be completely excluded from growth. Even cells infected with T2 phage did not cause additional breakdown of superinfecting T7. Thus the superinfection breakdown phenomenon is confined to a limited number of phage infections and does not provide a general explanation for mutual exclusion even among the T phages. The full significance of this breakdown reaction is not yet apparent and must be left for future work to decide.

In view of the phenomena of genetic recombination, multiplicity reactivation of ultra-violet killed phage and the complex breakdown reactions undergone by the infecting particle, it is unlikely that phage multiplication takes place by a process of binary fission. It is, however, easier to

dispose of this hypothesis than it is to substitute another. At the present time so little information is available that any explanation must be in the most general terms and highly speculative in addition. As a working hypothesis, the author visualizes the process as follows:

Immediately the phage is attached irreversibly to the cell a transformation occurs in which both phage and cell lose their identity and a new complex, the infected cell, is formed. The genetic determinants are supplied by the virus, the metabolic machinery is supplied by the cell, and the new complex proceeds to synthesize nucleoprotein which will later appear as virus particles. No material indispensable for formation of virus is supplied either by cellular constituents or by the infecting particle, but parts of each are used since they are present in a readily available form at the right time. The rest of the new virus substance is synthesized de novo from components present in the nutrient medium.

Thus presented phage reproduction involves two of the outstanding problems in biology -

(1) What is the nature of the forces that control cellular reactions; how is the direction of cellular metabolism passed from control by the cell to the determining influence of the infecting phage particle; how are the characteristics of the invading particle inherited by the progeny? It is impossible at present to answer any one of these questions.

(2) What is the mechanism of synthesis of nucleic acid and protein? The phage particle is represented as the

terminal point in a series of synthetic reactions. It contains the biological specificity and has the same chemical composition as the parent. Thus in a given infection the synthesis always leads to predominantly the same type of nucleoprotein and present indications are that this synthesis is the main metabolic function of the infected cell. Since phage systems are relatively easy to handle and can be studied under a variety of controlled conditions, they should represent ideal experimental material for investigation of nucleic acid and protein formation.

In studying the phage-cell interaction numerous experimental approaches to these problems may be devised. As an extension of the present work, the one that occurs most readily is an investigation into the uptake by growing phage of various C^{14} and S^{35} labelled compounds placed in the medium. Knowledge of the intracellular changes undergone by each compound before incorporation of the isotope into the virus should give some insight into the steps involved in nucleoprotein formation. When the virus is obtained with these isotopes in known positions in the structure it could be used to infect the host cell. A study of the fate of the labels should extend the knowledge gained in the present work and may help to answer the very important question whether material transfer is necessary for the inheritance of genetic characters by the viral progeny.

As was mentioned earlier, it is difficult at present to estimate how much of the knowledge gained with bacterial

viruses will apply to other types of virus. It would appear that many workers studying plant viruses have come to the conclusion that the virus merges with the cell to produce a completely new metabolic pattern in which the infecting particle loses its identity (Kennedy, Nature, 168, 890, 1951). This is a view similar to that held by many workers in the phage field. In the animal virus field there are many similarities between the behaviour of these viruses and the phages. Thus the adsorption of mumps and influenza viruses to the susceptible cells has been demonstrated (Hirst, J. Exptl. Med. 78, 99, 1943). Influenza virus has been shown to grow in a stepwise fashion like the phages with a latent period of 6 to 8 hours and an average burst size of about 80 particles per cell (Henle, Henle and Rosenberg, J. Exptl. Med. 86, 423, 1947). The work of Hoyle (Brit. J. Exptl. Path. 29, 390, 1948) suggests a breakdown of the infecting influenza particle. Burnet and Lind (J. Gen. Microbiol. 5, 67, 1951) have demonstrated the recombination of genetic characters between different strains of influenza virus and Henle and Liu (J. Exptl. Med. 94, 305, 1951) have found that infection of the host with ultra-violet killed influenza virus may result in multiplicity reactivation. In addition, mutual exclusion between many different animal viruses has been observed (Henle, J. Immunol. 64, 203, 1950).

It would therefore seem that many of the characteristics of the phages are possessed also by some of the animal viruses. The hope may not be ill-founded that phages serve as

useful model systems for investigation of the general problem of virus growth, and that many of the experimental techniques developed for phages may prove useful in other virus work.